Identification of the gene and neurons that control copulation in Drosophila

Identification of the gene and neurons that control copulation in *Drosophila* (ショウジョウバエの交尾を制御する遺伝子とニューロンの同定)

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ABSTRACT

Introduction The *platonic* (*plt*) mutant males of *Drosophila melanogaster* vigorously court females as the wild-type males do, but are unsuccessful in copulation. The mutants also show a morphological phenotype of short posterior-most longitudinal wing veins (L5). In this study, I aimed to identify the gene responsible for the *plt* mutation and the neural mechanism whereby copulation is impaired in *plt*.

Results It was found that the males heterozygous for *plt* and chromosomal deficiencies that delete a part of 55C region of the second chromosome show low copulation success and short L5 vein phenotypes. Within the region to which *plt* is mapped, there is a gene called *scribbler* (*sbb*), which encodes a transcription factor functioning downstream of *decapentaplegic* (*dpp*). When *sbb* mutant alleles were made heterozygous with *plt*, the flies also exhibited the short L5 vein and low copulation success. The *plt* mutation was induced by an insertion of the transposon *P* element, which tagged the gene in question. The inverse PCR technique located the *P*-element insertion site in the *sbb* locus: 31 bp downstream of transcription start site of *sbb*. RNA interference (RNAi) experiments were carried out to see if *sbb* knockdown recapitulates the *plt* phenotypes, by expressing genetically encoded RNAi constructs (*UAS-sbb RNAi*) in male flies using several different GAL4 drivers. When a heat-inducible GAL4 driver (*hs-GAL4*) was used, and the heat shock was applied at the larval stage, about 70% of emerged flies showed the

short wing vein phenotype. When the heat shock was applied at the pupal stage, about 10% of emerged flies showed the short wing vein phenotype. The copulation success rate was also decreased when the expression of *UAS-sbb RNAi* was induced at the larval stage, but not in the pupal stage. To examine if the *sbb* function is required in neurons for normal copulation behavior, we expressed *UAS-sbb RNAi* using a pan-neural GAL4 driver ($elav^{C155}$). *UAS-sbb RNAi* was expressed by two GAL4 drivers that drive expression in specific neurons in which *fruitless* (*fru*; fru^{NP21}) or *doublesex* (*dsx*; dsx^{GAL4}) are expressed. *fru* and *dsx* have an important part of the neural circuit that controls sexual behavior in *Drosophila*. We observed a low rate of copulation success when *UAS-sbb RNAi* was expressed by $elav^{C155}$, and also by fru^{NP21}. In contrast, copulation was completely inhibited when *UAS-sbb RNAi* was expressed by dsx^{GAL4}. The result demonstrated that *plt* functions in a subset of *dsx* expressing neurons. To determine which subset of *dsx*-expressing neurons are critical for the regulation of copulation, I performed the intersectional experiment by using *tsh-GAL80* (*tsh* is expressed in the thoracic region) and brain-specific *Otd-FLP* to restrict neurons in which *sbb* is knocked down. I also used different GAL4 drivers that are specified by neurotransmitters in the other set of intersection experiments. The results indicated that *sbb* expression in the neurons called *dsx*-SN and *dsx*-TN2 and *dsx*-expressing neurons in the abdominal ganglia are is pivotal for copulation to occur. These neurons are likely to be GABAergic and serotoninergic.

Conclusion We concluded that *plt* is an allele of *sbb*, and the impairment of *sbb* function in the *plt* mutant is responsible for its non-copulating phenotype. The *sbb* is involved in

the *dpp* pathway, and has been shown to play roles in multiple aspects in the development including axon guidance during the visual system development. The results of our study are consistent with the hypothesis that *sbb* function is required in the GABAergic and serotoninergic *dsx*-expressing neurons in the abdominal ganglion during development for establishing the neural circuit that regulates copulation behavior in male flies.

INTRODUCTION

The mating behavior of *Drosophila melanogaster* provides an excellent model system to determine the genetic construction of behavioral elements. In this species, male courtship is largely innate and consists of a complex series of ordered behaviors (Bastock and Manning, 1955, Hall, 1994, Yamamoto et al., 1997). Courtship is initiated by the male with aligning himself to a target female from behind, relying on visual and olfactory inputs for orientation. Then, the male taps the female's abdomen with his foreleg to sense pheromonal cues for gender and species recognition, followed by wing vibration to produce a species-specific courtship song. The male acquires pheromonal cues by licking the female's genitalia, after which he will attempt to copulate. The female can accept the male or reject him by moving away or by other means for rejection. When the female is fully receptive, she opens her wings and vaginal plates to make it physically possible for a male to copulate with her. During copulation, the male remains mounted on the female for about 20 min, after which he releases the genital contact. Thus, courtship behavior of *D. melanogaster* is composed of sequential actions in which auditory, visual, and chemosensory signals are exchanged between the male and female. For through understanding of courtship behavior, individual components of the behavior need to be quantified and separated (Hall, 1994; Greenspan, 1995).

Mutations that affect mating behavior are potentially useful to dissect the underlying mechanism. There are mutations that affect mating behavior in *D.*

melanogaster indeed. For example, mutations in *courtless* (*col*; Yamamoto and Nakano, 1998), *cuckold* (*cuc*; Castrillon et al., 1993), or *he's not interested* (*hni*; Yamamoto et al., 1997) exhibit a decrease in male courtship activity, while mutations in *spinster* (*spin*; Suzuki et al., 1997), *chaste* (*cht*; Yamamoto et al., 1997), and *dissatisfaction* (*dsf*; Finley et al., 1997) result in decreased female receptivity. However, most of them are yet to be studied in detail.

In the present study, I focused on a behavioral mutant called *platonic* (*plt*). *plt* mutant males are characterized by the lack of copulation. Although the *plt* mutant males vigorously court females as the wild-type males, and show the sequence of mating behaviors including orienting, following, tapping, singing and licking, they are unsuccessful in copulation. In addition to the behavioral phenotype, *plt* mutants show a morphological phenotype of short posterior-most longitudinal wing veins (L5). Although, the gene for these phenotypes has not been molecularly identified. The *plt* mutation has been mapped to 55C the second chromosome (D. Yamamoto, unpublished data). There is a gene named *scribbler* (*sbb*) in same region of the second chromosome. *sbb* is known to be involved in the *dpp* pathway, with roles in axon guidance during the visual system development.

To understand the molecular and neural mechanisms in which *plt* might be involved for the regulation of copulation, we first focused our attention on the male-specific circuitry composed of cells that express *fruitless* (*fru*). The male-specific Fru protein is expressed in the peripheral (PNS) and central nervous system (CNS) of males, but not of females (Lee et al., 2000 and Usui-Aoki et al., 2000), although transcription of the *fru* gene occurs in both sexes (Ito et al., 1996 and Ryner et al., 1996). *fru*-expressing

clusters, P1 and P2b, were shown to trigger male courtship behavior (Kimura et al., 2008, Kohatsu et al., 2011). P1 neurons are the prime components of the neural circuitry that initiates male courtship (Kimura et al., 2008, Kohatsu et al., 2011). In the absence of Fru proteins, males fail to produce pulse song and never attempt copulation toward females (Villella et al., 2008). Females that were made to express Fru proteins show many components of male-specific courtship behavior. However, they court less than wild-type males do and never attempt copulation (Demir and Dickson, 2005). Thus, *fru*itless expression is not sufficient to accomplish normal male courtship behavior, suggesting that other genes are required for a complete male courtship behavior to occur.

This second gene is *doublesex* (*dsx*). Males lacking *dsx* court at low levels and fail to generate the sing song component of courtship song (Villella et al, 1996). Male-specific Dsx and Fru proteins are coexpressed in several regions that are pivotal for establishing male sexual behavior. Thus, both genes are required for the specification of neural systems that generate male sexual behavior (Rideout et al., 2007, Billeter et al., 2006, Kimura et al., 2008).

Our hypothesis is that *plt* function is required in *fruitless* and/or *doublesex* expressing neurons for the normal copulation behavior. This study aimed to molecularly identify the gene for the *plt* mutation, and determine how this gene is involved in the mating behavior of males. The intriguing possibility is that *plt* functions in *fru* and/or *dsx* expressing cells to form the circuitry for the control of copulation by guiding neurites for proper destination. This hypothesis will be tested experimentally in the following sections.

MATERIALS AND METHODS

Drosophila **stocks**

All *Drosophila* cultures were maintained at 25˚C on a medium containing yeast, cornmeal, glucose, and agar. The following strains were established, or kept as lab stocks in our laboratory:

The wild type *Canton-S*, *+; plt/SM1; ry^{506}, +/w; plt/CyO; Sb/TM3 Ser*, *elav^{C155} w^{1118}*, *fru^{GAL4}*, *N630 hs-GAL4*, and *w; fru^{NP21}/TM3 Ser*.

The following strains are kind gifts of Dr. Tetsuya Tabata of the University of Tokyo:

yw; FRT42 sbb^{6}/CyO Act-GFP, yw; sbb^{k00702}/CyO, and *P{GT1}sbb^{BG01610}/CyO*.

w, ppk-GAL4 is a gift of Dr. Kazuo Emoto of the University of Tokyo.

w; Otd-FLP is a kind gift of Dr. David Anderson of the California institute of Technology.

w; dsx^{GAL4}/TM6, dsx^{FLP}/TM3, and *elav-GAL80* are kind gifts of Dr. Stephen F. Goodwin of the University of Oxford.

fru^{FLP} is a kind gift of Dr. Barry J. Dickson of the Janelia Farm Research Campus.

fru^{LexA} is a kind gift of Dr. Bruce S. Baker of the Janelia Farm Research Campus.

tsh-GAL80 is a kind gift of Dr. J. Simpson of the Janelia Farm Research Campus.

Repo-GAL80 is a kind gift of Dr. Leslie B. Vosshall of the Rockefeller University.

The following strains were obtained from the Bloomington Drosphila Stock Center:

w^{1118}; Df(2R)ED3610/CyO amos^{Roi-1}, w^{1118}; Df(2R)BSC334/CyO, w^{1118}; Df(2R)BSC337/CyO, w^{1118}; Df(2R)ED3683/SM6a, w^{1118}; Df(2R)BSC335/CyO, w^{1118}; Df(2R)Excel7153/CyO, w^{1118}; Df(2R)BSC339/CyO, w^{1118}; Df(2R)BSC483/CyO, w; Tdc2-GAL4, w; ple (TH)-GAL4, w, VGlut-GAL4, w^{1118}; VGlut^{OK371}-GAL4, w;Cha-GAL4, w; Trh-GAL4, w; GAD-GAL4 (II), GAD-GAL4 (III),w; poxn-GAL4/CyO, w;UAS-Dcr2 (III), tubP>GAL80>; Bl/CyO; TM2/TM6, w; tubP>GAL80>/CyO; TM2/TM6, w; Sp/CyO; tubP>GAL80>/TM6B, w; tubP>stop>GAL80>; MKRS/TM6, w; wg^{Sp-1}/CyO; tubP>stop>GAL80>, UAS-mCD8::GFP, UAS>stop>mCD8::GFP, LexAop-GAL80.

w^{1118}; P{GD10734}v41845 strain was obtained from the Vienna Drosophila RNAi Center.

Behavioral Assays

The virgin males and females were collected at eclosion. Males were placed singly in food vials, while 10 females were placed together in single food vials. They were kept at 25˚C 12 hr/12 hr of light/dark cycle in an incubator (SANYO) until behavioral assays. Behavioral assays were carried out on males aged for 6–7 days after eclosion. The male was placed in a small chamber with a wild-type virgin female (5–6 days after eclosion). The mating behavior was recorded for 60 minutes (min) by the Watec-232S camera. The courtship activity was measured by the courtship index defined by the following equation;

$$\frac{\text{The courtship duration}}{\text{The total observation time}} \times 100$$

The courtship duration is the duration in which the male shows any element of the courtship behavior including orientation, following, tapping, wing extension, licking, and attempted copulation. The observation period started just after the introduction of the virgin female into the observation chamber. The observation time for the courtship index calculation was 10 min after the start of observation, or until copulation occurred within the 10 min observation period. The mating success was calculated by the number of pairs that copulated during the observation period (60 min) as divided by the number of total pairs observed. The statistical analyses were carried out by the ANOVA followed by the Mann-Whitney`s U tests for the courtship index, or by the Chi-square test for the mating success.

Genetically encoded RNAi

To express genetically encoded RNAi constructs, I used the GAL4-UAS system (Brand and Perrimon, 1993). In the GAL4-UAS system, a GAL4 line that exhibits a desired expression pattern is used to activate a target gene specifically in a particular cell or tissue. The target gene is placed under the control of the target sequence of GAL4 named *UAS*. In this study, five different GAL4 drivers ($elav^{C155}$, fru^{GAL4}, *hs-GAL4,* $fru^{NP21}$and dsx^{GAL4}) were used to express a *UAS-sbb RNAi* construct, *P{GD10734}v41845*. To apply heat shock, larvae or pupae in food vials were placed in a programmable temperature-controlled incubator (TAITEC) and were exposed to the heat shock treatment of 37˚C for 45 min or 1 hour. Flies were typically subjected to 3-time heat shocks; flies received a heat shock for 45 min or 1 hour every consecutive three day.

Intersectional Experiments

To restrict GAL4 expression in the tissue of interest, the ET-FLP-induced intersectional GAL80/GAL4 repression (FINGR) method was used. The FINGR method has three components: 1) the GAL4/UAS system, 2) FLP/FRT-mediated GAL80 converting tools, and 3) enhancer-trap FLP (ET-FLP). GAL4 expression can be restricted by using two complementary GAL80-converting tools: *tubP>GAL80>* (flip out) and *tubP>stop>GAL80>* (flip in). In the flip-out approach, GAL80 will relieve GAL4 repression in cells in which GAL4 and FLP overlap. In the flip-in approach, GAL80 will suppress GAL4 expression wherever GAL4 and ET-FLP intersect (Bohm et al., 2010; Fore et al., 2011).

When *tubulin-FRT-GAL80-FRT-stop* (*tubP>GAL80>stop*) is combined with *Otd-FLP* that specifically expresses FLP in the brain, the *GAL80* is flipped out by *Otd-FLP* and hence *UAS-sbb RNAi* is expressed in the *dsx-GAL4 and Otd-FLP* intersecting region. When *tubulin-FRT-stop-FRT-GAL80* (*tubP>stop>GAL80>*) is combined with *Otd-FLP, GAL80* is flipped in, *dsx-GAL4* activity repressed and *UAS-sbb RNAi* is not expressed where *dsx-GAL4* and *Otd-FLP* intersect.

Wing preparation

Flies were dipped for 1-2 minutes in 10 ml of ethanol to remove the wax on the surface, and the wings were removed from the body by forceps. The wings were transferred to 10 ml of phosphate buffered saline (PBS), then mounted in 80% glycerol in PBS on a slide glass. Images of the wing were captured using a fluorescence dissection microscope (Leica M205FA).

Immunohistochemistry

The central nervous system (CNS) was dissected from flies that were reared at 25°C and aged for 8-10 days in PBS and the CNS was immersed in 4% paraformaldehyde in PBS for 60 min. The CNS was the washed three times in PBT (PBS supplemented with 0.4% Triton-X), thereafter, the reaction was blocked 10% Normal Goat Serum (NGS). Then, the tissue was incubated with the primary and secondary antibodies for 3 days at 4°C. Primary antibodies used in this study were rabbit anti-GABA diluted at 1:500 (Sigma), rabbit anti-5HT at 1:500 (Sigma), chicken anti-GFP at 1:1000 (abcam), and rabbit polyclonal anti-GFP at 1:1000 (Molecular Probes), and mouse monoclonal nc82 at 1:20 (DSHB, University of lowa, IA). Secondary antibodies used were: Alexa647-conjugated goat anti-mouse IgG at 1:200, Alexa546-conjugated goat anti-rabbit IgG at 1:200, Alexa488-conjugated goat anti-chicken IgG at 1:200, Alexa488-conjugated goat anti-rabbit IgG at 1:500, and Alexa546-conjugated goat anti-mouse IgG at 1:500 (all from Invitrogen). All samples were mounted in VectaShield. Stacks of optical sections were obtained with a Zeiss LSM 510 META confocal microscope and were processed with ImajeJ software.

Inverse PCR

1. **Digestion by Restriction Enzymes**

The HinP1I (New England Biolabs R01245) and Hpa II (New England Biolabs R01715) restriction enzymes were used to digest the genomic DNA (Table 1). The NEB 4 reaction buffer was used for HinP1 I, and the NEB 1 reaction buffer was used for Hpa II.

Table 1. Preparation of DNA digestion

Genomic DNA (~2 flies)	10 µl
10X Reaction buffer (NEB 1 or NEB 4)	2.5 µl
Restriction enzyme (HinP1 I or Hpa II)	0.5 µl
H_2O	12 µl
Total	25 µl

The reactions were carried out at 37˚C for 2.5 hrs. Then, the enzymes were heat inactivated by incubating at 70˚C for 20 min. The digested DNA was stored at -20 ˚C.

NEB 1 Buffer Components

10mM Bis-Tris-Propane-HCl
10mM $MgCl_2$
1mM Dithiothreitol
pH 7.0 (25°C)

NEB 4 Buffer Components

50mM Potassium Acetate
20mM Tris-acetate
10mM Magnesium Acetate
1mM Dithiothreitol
pH 7.9 (25°C)

2. Ligation

The digested genomic DNA is circularized by self-ligation in a ligation solution (Table 2) by the following steps:

Table 2. The composition of DNA ligation solution

Digested genomic DNA	10 µl
10X Ligation buffer with ATP	40 µl
T4 DNA ligase (NEB)	2 µl
H_2O	348 µl
Total	400 µl

1) Incubation at 4˚C for overnight.
2) Addition of 40 µl of 3 M sodium acetate (CH_3COONa: NaOAc; Table 3) and 1 ml of 70% ethanol. Followed by mixing.
3) Incubation at -20 ˚C for at least 10 min. Centrifugation at the maximal speed (12,000-15,000 rpm) at 4˚C for 30 minutes. Discarding the supernatant.
4) Addition of 500 µl of 70% ethanol, followed by brief mixing, centrifugation at the maximal speed at 4˚C for 15 min, and discarding the supernatant.
5) Air-drying the pellet for 30 min followed by re-suspending the pellet in 150 µl of H_2O.
6) Storage at -20˚C.

Reagent: **Table 3.** 3 M sodium acetate (NaOAc) solution

3 M NaOAc	
NaOAc	40.824 g
H_2O	80 ml
Glacial acetic acid	~12 ml
Total	100 ml

1) Dissolving NaOAc in water.
2) Adjusting pH to 5.2 with glacial acetic acid, and adjusting the volume to 100 ml with water.
3) Autoclaving.

3. PCR

The EY.3.F and EY.3.R primers were used to amplify the flanking DNA to the 3' end of the *P* element, while Plac4 and Plac1 primers were used to amplify that to the 5' end of the *P* element (Table 4). PCR was performed (Table 6) with gradient annealing temperatures to determine the optimum annealing temperature. The PCR was carried out in the C1000 Thermal Cycler (BioRad).

Table 4. The sequences of the primers:

For 5'P:

Plac4 (ACTGTGCGTTAGGTCCTGTTCATTGTT)

Plac1 (CACCCAAGGCTCTGCTCCCACAAT)

For 3'P:

EY.3.F (CCTTTCACTCGCACTTATTG)

EY.3.R (GTGAGACAGCGATATGATTGT)

Table 5. Preparations of PCR

Template (ligated genomic DNA)	5 µl
2 mM each dNTP (Toyobo)	2 µl
10 X Reaction buffer (10 X Ex Tag Buffer, Takara)	2.5 µl
10 µM forward primer (Plac 4 or EY.3.F)	0.5 µl
10 µM reverse primer (Plac 1 or EY.3.R)	0.5 µl
Polymerase (Ex Taq HS 20, Takara RR006)	0.2 µl
H_2O	14.3 µl
Total	25 µl

Table 6. Thermal cycle for PCR

95˚C	30 sec	
95˚C	30 sec	
50-65˚C	30 sec	35 cycles
72˚C	60 sec	
72˚C	5 min	
4˚C	Hold	

After PCR, 10µl of the PCR product was mixed with 1 µl of 10 x loading buffer, and electrophoresed on a 1.5% agarose gel at 100 V for about 25-30 min. The gel was stained by an ethidium bromide solution for 25 min. The gel was photographed on the Benchtop 2UV Transilluminator (MITSUBISHI).

4. Nucleotide Sequencing

Prior to sequencing, Qiagen PCR purification kit (P/N 28104) was used to purify double-stranded DNA fragments from primers, nucleotides, polymerases and salts. Also Qiagen gel extraction kit (P/N 20021) was used to purify double-stranded DNA fragments from the excised bands in gels. Sp1 sequencing primer (Table 7) was used for 5' end of the *P*-element (Plac1-Plac4) and 3'P Seq sequencing primer (Table 7) was used for 3' end of the *P*-element (EY.3.F- EY.3.R). The sequencing was performed by 3500 Genetic Analyzer (Applied Biosystems, HITACHI).

Table 7. The sequences of the primers:

Sp1 (ACA CAA CCT TTC CTC TCA ACA A)

3'P Seq (GAG CGT GAA TAA CGT TCG TA)

RESULTS

Genetic complementation tests

In a preceding study in our laboratory, the *plt* mutation has been mapped to 55C on the second chromosome (D. Yamamoto, unpublished data). To map the mutation at a higher resolution, genetic complementation tests were performed by using chromosomal deficiencies and mutations in this region (Figure 1). The red bars in Figure 1 indicate the deleted segments in the deficiencies. I performed genetic complementation tests by placing one of these deficiency chromosomes in *trans* to the homologous chromosome harboring the *plt* allele. Some of such *plt* hemizygotes exhibited short wing vein phenotype at high frequencies (Figure 2A); e.g., *plt* hemizygotes over *Df(2R)ED3610, Df(2R)BSC334* or *Df(2R)ED3683*. In contrast, the normal wing vein pattern (Figure 2A) was observed in hemizygotes with *Df(2R)BSC483, Df(2R)Excel7153, Df(2R)BSC335* or *Df(2R)BSC339* (Figure 2B). The behavioral phenotype was paralleled with the wing vein phenotype. The deficiencies that failed to complement the wing vein phenotype (i.e., *Df(2R)BSC334* and *Df(2R)ED3683*) also failed to complement the no-copulation phenotype of *plt*, while the deficiencies that complemented the wing vein phenotype (i.e., *Df(2R)BSC483, Df(2R)Excel7153, Df(2R)BSC335* and *Df(2R)BSC339*) also complemented the behavioral phenotype (Figure 3). The result indicates that the *plt* mutation exists within the small region between the proximal breakpoint of *Df(2R)ED3683* and the distal breakpoint of *Df(2R)BSC334* (Figure 1). Within this region,

there is a gene called *sbb*. We examined if mutant alleles of *sbb* complement the *plt* phenotypes. Double heterozygotes of *plt* and $sbb^{BG01610}$, sbb^{6} or sbb^{k00702} all showed the short wing vein phenotype at high frequencies (Figure 2). They also showed the no-copulation phenotype (Figure 3) while exhibiting high courtship indices (Figure 4). The genetic complementation tests strongly suggest that *plt* is an allele of *sbb*.

Determination of the *P*-element insertion site in the *plt* mutant

I used the inverse PCR technique to find the *P*-element insertion site in the *plt* mutant genome. In an inverse PCR, genomic DNA flanking to a region with known nucleotide sequence (in this case the *P* element sequence) is amplified. The template genomic DNA is digested with a restriction enzyme, circularized by self-ligation, and amplified with primers complementary to the known sequence. In this study, genomic DNA of the *plt* mutant and, as a negative control, the wild-type *Canton-S* was prepared. Nucleotide sequences of the amplified DNA fragments were determined by direct sequencing of the PCR fragments. In the search of the *Drosophila* genomic DNA database with the determined nucleotide sequences, both sequences obtained from 5'P and 3'P ends indicated that the *P*-element insertion site is in the *sbb* locus, 31-bp downstream of its transcription start site (Figure 5).

Knocking down of *sbb* by genetically encoded RNAi

To further confirm the molecular identity of *plt*, and at the same time, to explore the spatial and temporal requirements of *sbb* gene function for mating behavior, RNA

interference (RNAi) experiments were carried out by expressing genetically encoded RNAi constructs using the *GAL4-UAS* system (Brand and Perrimon, 1993).

First, I used a heat-inducible GAL4 driver, *hs-GAL4*, to drive the expression of *UAS-sbb RNAi*. Heat shocks were applied at several different timings during development. When the heat shock was applied at 3 days after egg laying (AEL the early larval stage), the emerged flies did not show the short wing vein phenotype. When the heat shock was applied at 4 days or 5 days AEL (the late larval stage), 70% and 23% of emerged flies showed the short wing vein phenotype, respectively. When the heat shock was applied at 7 days AEL (the pupal stage), 10 % of emerged flies showed the short wing phenotype. Two negative controls were prepared: flies carrying only *hs-GAL4* (*hs-GAL4/+*) and flies carrying only *UAS-sbb RNAi* (*UAS-sbb RNAi/+*). The *hs-GAL4/+* flies were heat shocked at larval or pupal stages. In the negative controls, abnormal wing veins or positions were not observed (Figure 6). As to the behavioral phenotype, mating success was decreased when the expression of *UAS-sbb RNAi* was induced by applying heat shock at the late larval stage, but not when the heat shock was applied at the pupal stage (Figure 7).

To examine if *sbb* function is required in neurons for the normal copulation behavior, *UAS-sbb RNAi* was expressed by the pan-neural GAL4 driver $elav^{C155}$ in the presence and absence of *UAS-Dicer2* to enhance transgenic RNAi effects (Dietzl et al., 2007). Mating successes of flies that had both $elav^{C155}$ and *UAS-sbb RNAi* in the presence and absence of *UAS-Dicer2* were lower than those of the negative controls (Figure 8A). There were no significant differences between test flies and their negative controls in the

courtship index (Figures 8B). The result indicates that *sbb* function is required in neurons for attaining the normal copulation success rate.

Knockdown of *sbb* in the sexually dimorphic neurons

It has been shown that a significant part of neural circuits that control sexual behaviors in *Drosophila* is composed of neurons that express the *fru* gene (Ito et al., 1996, Ryner et al., 1996; Baker et al., 2001; Yamamoto and Nakano, 1998). Next, I asked whether *sbb* function is required in these *fru*-expressing neurons for the normal copulation behavior. To this end, I expressed *UAS-sbb RNAi* using the GAL4 drivers that drive expression in the *fru*-expressing neurons, fru^{GAL4} and fru^{NP21} (Manoli et al., 2005, Stockinger et al., 2005, Kimura et al., 2005) with or without *UAS-Dcr2*. When fru^{GAL4} was used to drive the expression of *UAS-sbb RNAi*, mating successes were lower than those of control groups (Figure 9A). The courtship indices were normal between test flies and fru^{GAL4} or *UAS-sbb RNAi*, two negative controls. When *UAS-sbb RNAi* was expressed by fru^{NP21}, one of GAL4 enhancer trap lines with an insertion in the *fru* locus, in the presence and absence of *UAS-Dicer2*, mating successes were significantly decreased when compaired with those of controls. These males showed a remarkably low mating success rate of 43% (Figure 10A). There was no significant difference in courtship indices between fru^{NP21} *UAS-sbb RNAi* and respective negative controls (Figure 10B). This result suggests that the neurons that require *sbb* function for normal copulation behavior contain *fruitless*-expressing neurons.

Next, I asked whether *sbb* function is required in *dsx*-expressing neurons, because not only *fru* but also *dsx* are involved in establishing sexual features of the neural

circuitry and behavior in fruit flies (Shirangi et al., 2006, Kimura et al., 2008, Robinett et al., 2010, Rideout et al., 2010, Yamamoto and Koganezawa, 2013, Pan et al, 2014). *dsx*^{GAL4} is expressed in neural and non-neural cells (Robinett et al., 2010, Rideout et al., 2010). I visualized *dsx*^{GAL4} expression in the CNS of a male brain using the *UAS-mCD8::GFP* membrane-bound GFP reporter. Neural clusters labeled in the brain included sexually dimorphic *dsx*-pC1 and *dsx*-pC2l that lie in the dorsal inferomedial and inferolateral protocerebrum surrounding the mushroom body calyces. In the suboesophageal ganglion (SOG) two male-specific neurons, *dsx*-SN, were labeled. In the thoracic ganglia (TG), *dsx*^{Gal4} expression in the male-specific *dsx*-TN1 and *dsx*-TN2 neuronal clusters were observed. *dsx*-TN1 neurons were located in the prothoracic ganglion (pr), whereas unpaired or paired *dsx*-TN2 neurons were located in the mesothoracic (ms) or metathoracic (mt) ganglion. A large number of unclassified *dsx*^{GAL4} positive neurons are in the abdominal ganglion (Figure 11A). To examine the function of *sbb* in the *dsx*-expressing neurons, *UAS-sbb RNAi* was expressed by *dsx*^{GAL4} in the presence and absence of *UAS-Dcr2*. Strikingly, the flies with *sbb*-knockdown did not copulate (Figure 11B) although the courtship indexes were practically normal in these flies (Figure 11C). I used the heterozygotes *dsx*^{GAL4}*, Dcr2* flies with *Canton-S* and *UAS-sbb RNAi* with *Canton-S* for the negative controls. Any of the test and control groups were normal in the courtship index (Figure 11B-C). These results demonstrate that *sbb* function is required in the *dsx*-expressing neurons for normal copulation behavior.

To determine the *sbb* requirement in *dsx* and/or *fru* expressing neurons for normal copulation, I attempted *sbb* knockdown in three groups of neurons, i.e., *fru*/*dsx* double positive, *fru* single positive and *dsx* single positive.

First, I examined the effect of *sbb* knockdown in *fru*/*dsx* double positive cells. Indeed, recent studies have suggested that *dsx* and *fru* collaborate to control development of specific neural substrates for male sexual behavior and some of the neurons composing these circuitries coexpress male specific Dsx^M and Fru^M proteins (Billeter et al., 2006, Rideout et al., 2007, Kimura et al., 2008). To determine the contribution of *fru* and *dsx* double positive neurons in copulation behavior, I performed the intersectional knockdown experiment with fru^{FLP} that flipped out GAL80 from the *tubP>GAL80>* cassette and hence allowed *UAS-sbb RNAi* to be expressed in the dsx^{GAL4} and fru^{FLP} intersecting cells (Figure 12A; Yu et al., 2010, Bohm et al., 2010, Fore et al., 2011). I found that *sbb* knockdown in *fru* and *dsx* double positive neurons had no effect on copulation (Figure 12B) and courtship activities (Figure 12C). This result indicates that *sbb* function in *fru* and *dsx* double positive neurons are dispensable for normal copulation.

Next, I examined the possible contribution of *fru* single positive neurons (Figure 13A). It has been shown that, in the absence of Fru^M proteins, males fail to produce the pulse song and attempt copulation (Villella et al., 2008). Conversely, females expressing Fru^M display many male-specific courtship behaviors (Demir and Dickson, 2005, Rideout et al., 2007). The flipping out of the stop cassette sequence in tubP>stop>GAL80 by the FLP activity of dsx^{FLP} (Rezaval et al., 2014) enables the expression of GAL80 only in the *dsx*-expressing cells (Bohm et al., 2010, Fore et al., 2011). As a result, *UAS-sbb RNAi* is expressed only in *fru* single positive cells. In this intersectional *sbb* knockdown, all experimental flies copulated (Figure 13B) and gave a high courtship index (Figure 13C) as contrasted with *sbb* knockdown in all *fru*-expressing neurons, which yielded reduced

copulation success (Figures 9A-10A). These results seem to indicate that *dsx* single positive neurons contribute to *sbb*-dependent copulation behavior.

Therefore, I performed yet another intersectional *sbb* knockdown experiment in *dsx* single positive neurons by combining fru^{LexA} and *LexAop-GAL80* with dsx^{GAL4} (Figure 14A; Little and Mount, 1982, Butala et al., 2008). In this intersectional *sbb* knockdown, experimental flies did not copulate (Figure 14B), while the courtship activity was high (Figure 14C). This result supports the notion that *fru*-negative and *dsx*-positive neurons play a primary role in *sbb* dependent copulation behavior.

dsx-GAL4 is expressed not only in neurons but also in non-neural cells, including male internal genitalia, proboscis, maxillary palps, trochanter, coxa, femur, tibia, mid-gut, rectum and others (Robinett et al., 2010, Rideout et al., 2010). To evaluate the possible involvement of non-neural *sbb*-expressing cells in the non-copulating phenotype in *sbb*-deficient flies, I performed an intersectional experiment with dsx^{GAL4} and *UAS-sbb RNAi* in the presence of *elav-GAL80* (Rideout et al., 2010), which suppresses GAL4 function only in the neurons (Figure 15A). All of experimental flies with non-neural *sbb* knockdown copulated (Figure 15B) and gave a high courtship index (Figure 15C). This result indicates that *sbb* functions in *dsx*-expressing non-neural tissues are dispensable for the control of copulation. Therefore, *sbb* expression in the *dsx*-expressing "neurons" must be critical for copulation.

In addition, I performed an intersectional experiment with dsx^{GAL4} and *UAS-sbb RNAi* in the presence of *Repo-GAL80* (*Repo* is expressed only in the glial cells; Crickmore and Vosshall, 2013) to check if glial cells contribute to the non-copulation phenotype in *sbb*-deficient males. When *sbb* knockdown was accomplished in non-glial

dsx^{GAL4} expressing cells (Figure 16A), all of experimental flies failed to copulate (Figure 16B), while the courtship activity was normal (Figure 16C). This result indicates that glial cells do not contribute to the non-copulating phenotype in *sbb* deficient flies.

sbb **knockdown in subsets of** ***dsx*****-expressing neurons**

To learn which part of *dsx*-expressing neurons is important for copulation, I performed an intersectional experiment, in which *UAS-sbb RNAi* expression *via dsx*^{GAL4} was excluded from the thorax by *teashirt-GAL80* (*tsh-GAL80)* (*tsh* is expressed only in the thoracic region; Fasano et al., 1991, Roder et al., 1992). As a result, *sbb* knockdown was restricted to: *dsx*-pC1, *dsx*-pC2l and *dsx*-SN neurons in the brain, *dsx*-TN2 neurons and peripheral sensory afferents in the thorax and abdominal neurons. The reason why the thoracic *dsx*-TN2 neurons are not affected by *tsh-GAL80* is not known. *dsx*-TN1 neurons in the thoracic ganglion are eliminated from the region in which *sbb* is knocked down (Figure 17A). While the experimental flies with *sbb* knockdown in TN2 neurons and non-thoracic tissues did not copulate (Figure 17B), while showing a high courtship activity (Figure 17C). This result indicates that *sbb* expression in the *dsx*-TN1 neurons is not essential for the execution of copulation. This result also means that *sbb* expression in the *dsx*-TN2 neurons, *dsx*-expressing neurons in the brain or the abdominal ganglion must be critical for copulation.

To clarify the possible involvement of brain *dsx*-expressing neurons in the *sbb*-dependent control of copulation, I performed the intersectional experiment, in which brain-specific *Otd-FLP* (*otd* is expressed only in the central brain region; Asahina et al., 2014, Kimura et al., 2015) was used to knockdown *sbb* only within the brain *dsx-GAL4*

neurons. In this intersection, the *dsx*-SN, *dsx*-TN1, *dsx*-TN2 and *dsx*-abdominal neurons are negative for GAL4 expression. On the other hands, the *dsx*-expressing neurons in the brain including *dsx*-pC1 and *dsx*-pC2l neurons express GAL4 (Figure 18A). Experimental flies in which *sbb* is knocked down only in the brain *dsx* neurons showed the high copulation success (Figure 18B) and courtship index (Figure 18C). This result strongly suggests that *dsx*-expressing neurons in the brain do not contribute to *sbb*-dependent copulation behavior.

To investigate only *dsx*-expressing neurons outside the brain for their role in copulation, I performed the intersectional experiment, in which brain-specific *Otd-FLP* was used to repress dsx^{GAL4} via *tubP >stop>GAL80* (Bohm et al., 2010, Fore et al., 2011). In this case, the brain region including *dsx*-pC1 and *dsx*-pC2l are negative for GAL4 expression. On the other hands, the *dsx*-SN in the SOG, *dsx*-TN1, *dsx*-TN2 and peripheral sensory afferents in the thorax and *dsx*-abdominal neurons are positive for GAL4 expression (Figure 19A). In this intersectional *sbb* knockdown, the experimental flies with no *sbb-RNAi* expression in the brain did not copulate (Figure 19B) while they gave a high courtship index (Figure 19C). This result suggests that *dsx*-SN, *dsx*-TN1, *dsx*-TN2 and *dsx*-abdominal neurons are candidate cells that mediate *sbb*-dependent copulation behavior. Among these, *dsx*-TN1 can be excluded from the candidates, based on the result shown in Figures 17A-B. Therefore, in conclusion, *dsx*-SN, *dsx*-TN2 and *dsx*-abdominal neurons remain to be candidates for the sites where *sbb* acts for copulation regulation.

Non-cholinergic *dsx*-expressing neurons are important to control copulation

I performed the intersectional experiment with *dsx-GAL4* and *UAS-sbb RNAi* in the presence of *Cha-GAL80* which inhibits GAL4 function in the cholinergic neurons (Kitamoto, 2002). This experiment was done because acetylcholine is a major excitatory neurotransmitter in *Drosophila* CNS (Yasuyama and Salveterra, 1999) and a large portion of *dsx* neurons are thought to be cholinergic neurons. In the presence of *Cha-GAL80*, *dsx*-pC1, *dsx*-pC2l and *dsx*-TN1 neurons are negative for GAL4 expression. *dsx*-SN, a subset of *dsx*-TN2, whereas *dsx*-abdominal neurons are positive for GAL4 expression (Figure 20A). Strikingly, experimental flies carrying *Cha-GAL80* did not copulate (Figure 20B) but showed a high courtship activity (Figure 20C). Here, *sbb* knockdown took place in the *dsx*-SN, *dsx*-TN2, *dsx*-abdominal neurons and peripheral sensory afferents projecting to the thoracic ganglion. These results indicate that non-cholinergic *dsx*-expressing neurons primarily control the *sbb*-dependent copulation behavior.

***dsx*-expressing chemosensory neurons partially contribute to *sbb*-dependent copulation control**

To examine the possible involvement of *dsx*-expressing peripheral afferents in the thoracic ganglion, which contain both chemosensory and mechanosensory neurons, I carried out the intersectional *sbb* knockdown in *dsx* neurons that are also positive for *pox neuro-GAL4* (*poxn-GAL4*; Boll and Noll, 2002) and *pickpocket-GAL4* (*ppk-GAL4*; Liu et al., 2002, Figure 21A). Whereas the test flies with *ppk-GAL4* revealed high mating success (Figure 21B), the test flies with *poxn-GAL4* showed reduction in mating success.

A slight, yet statistically significant (Figure 21B). There were no significant differences between test flies and negative controls in the courtship index (Figure 21C). This result shows that the manipulation of chemosensory neurons, but not mechanosensory neurons, has a moderate effect on *sbb*-dependent copulation behavior.

Roles of non-cholinergic *dsx*-expressing neurons in *sbb*-dependent copulation control

I performed the intersectional *sbb* knockdown experiment by employing dsx^{FLP} in combination with different GAL4 drivers that are specified by neurotransmitters (Figure 22A). The test flies with a *tyrosine decarboxylase 2* (*Tdc2*)-*GAL4* driver that specifies octopamine producing cells (Cole et al., 2005) gave a high mating success and courtship index (Figure 22B-C). This result indicates that *sbb* expression in octopaminergic neurons is dispensable for controlling copulation behavior (Figure 22B). The mating successes were moderately decreased in test flies with *choline acetyltransferase* (*Cha)-GAL4* (Salveterra and Kitamoto, 2001), *tyrosine hydroxylase* (*TH)-GAL4* (Friggi-Grelin, 2003) and *vesicular glutamate transporter* (*Vglut)-GAL4* (Mahr and Aberle, 2006) (Figure 22B). These results indicate that cholinergic, dopaminergic and glutamatergic neurons likely require *sbb* functions in their regulation of copulation behavior.

Mating success was markedly decreased when dsx^{FLP} activated *glutamic acid decarboxylase GAD-GAL4* (Mehren and Griffth, 2006, Parisky et al., 2008) or *tryptophan hydroxylase* (*Trh)-GAL4* (Alekseyenko et al., 2010) to drive *UAS-sbb RNAi* (Figure 22B). There were no significant differences between test flies and negative controls in the courtship index (Figure 22C). These results demonstrate that GABAergic and

serotoninergic *dsx*-expressing neurons play a main role in *sbb*-dependent copulation control.

Given that *Trh-GAL4* and *GAD-GAL4* yield the strongest effect to reduce copulation, I carefully inspected the neurons that are targeted by these GAL4s with aid of dsx^{FLP} and the *UAS>stop>mCD8::GFP* reporter. The glutamatergic *dsx* neurons and the GABAergic *dsx* neurons are shown in Figure 23A and Figure 23B-C, respectively. In Figure 23B, *GAD-GAL4* on the 2nd chromosome was used whereas in Figure 23C, *GAD-2B-GAL4* on the 3rd chromosome was used. In these three images, the *dsx*-SN, *dsx*-TN2 and a subset of *dsx*-abdominal neurons are positive for marker expression. In addition, a small subsets of *dsx*-pC1, *dsx*-pC2l and *dsx*-TN1 neurons are positive. The serotoninergic *dsx* neurons are shown in Figure 23D. Only *dsx*-SN, *dsx*-TN1 and *dsx*-TN2 neurons as well as 8 abdominal neurons are positive for marker expression. The *dsx*-pC1 and *dsx*-pC2l neurons are negative for *Trh-GAL4* expression. These results demonstrate that the key neurons must be locating in the abdominal ganglion and are likely to be GABAergic or serotoninergic.

DISCUSSION

In the present study, I identified the *sbb* gene to be responsible for the *plt* mutation. I further searched for the neurons in which *sbb* functions for copulation.

plt is an allele of *sbb*

With genetic complementation tests using chromosomal deficiencies, I was able to map the *plt* mutation into a narrow region of the second chromosome that contains the *sbb* locus. When *sbb* mutant alleles were made heterozygous with *plt* or hemizygous with the deficiencies uncovering this locus, the flies showed similar behavioral and morphological phenotypes to those of the *plt* mutant. The *P*-element insertion site in the *plt* mutant was molecularly determined to be in the *sbb* locus 31-bp downstream of the transcription start site by means of inverse PCRs and subsequent DNA sequencing. Knocking down of *sbb* by genetically encoded RNAi constructs produced similar phenotypes to the *plt*-mutant phenotypes. All of these results indicate that the *plt* mutation is an allele of the *sbb* locus, and impairment of *sbb* function is responsible for the non-copulation phenotype of the *plt* mutant. Also, there was a significant reduction in numbers of attempted copulation in *plt* mutant male flies.

The *sbb* gene encodes a putative transcription factor that is involved in the Decapentaplegic (Dpp) signaling pathway (Funakoshi et al., 2001). Dpp belongs to the TGF-β family, and plays important roles in numerous contexts during development (Brummel et al., 1994; Nellen et al., 1994; Tanimoto et al., 2000). To gain an insight into

how the *sbb* function is involved in the copulation behavior of *Drosophila*, the spatial and temporal requirements of *sbb* were examined by RNAi experiments. When *sbb* was knocked down in all neurons by expressing *UAS-sbb RNAi* using the pan-neural GAL4 driver $elav^{GAL4}$, the mating success rate was decreased compared with that in controls. Similar to the *plt* mutant phenotype, decreased mating success was observed when *sbb* was knocked down at the larval stage by using the heat-inducible GAL4 driver *hs-GAL4*. These results suggest that the *sbb* function is required in neurons at the larval stage to establish the normal copulation behavior in the adult male. It is conceivable that *sbb* has a function in the development of particular neurons that are essential for normal copulation behavior. In fact, it has been reported that *sbb* is expressed in various tissues including larval neurons (Yang et al., 2000), and have functions in neuronal development. For example, a mutant allele of *sbb* shows a phenotype of abnormal axon guidance during the development of the visual system neurons (Senti et al., 2000; Rao et al., 2000). Another mutant allele of *sbb* shows a phenotype of an abnormal locomotion behavior of larvae, and the abnormal behavior is restored by expressing a wild-type *sbb* transgene (*UAS-sbb*) with the pan-neural GAL4 driver $elav^{GAL4}$ (Suster et al., 2004). Thus it is tempting to infer that *sbb* plays a role in the development of neurons for controlling copulation.

sbb **functions in** ***dsx*****-expressing neurons controlling copulation**

It has been shown that a large part of sexual behavior in *Drosophila* is controlled by neurons that express the *fru* gene (Ito et al., 1996; Ryner et al., 1996; Baker et al., 2001; Dickson, 2008; Yamamoto and Nakano, 1998). Fru protein is expressed in the male central and peripheral nervous system but not female nervous system. Fru confers sex-

specificity on morphology, and presumably physiology also, of neurons by regulating transcription of downstream genes. (Yamamoto and Koganezawa, 2013). First I examined if the non-copulation phenotype in *sbb* mutants is ascribable to some defects in *fru*-expressing neurons by knocking down *sbb* in these neurons. When *UAS-sbb RNAi* was expressed in the *fru*-expressing neurons by the fru^{GAL4} driver (Stockinger et al., 2005; Kimura et al., 2005), a weak but a significant decrease in the mating success was observed. This result suggests that some of *fru*-expressing neurons might require *sbb* function for controlling copulation.

In *Drosophila*, not only *fru* but also *dsx* play central roles in the sexual differentiation. *dsx* is the founder member of the conserved DMRT (Doublesex- and Mab-3-related transcription factor-like) family genes known to control the sex determination of many animals including the fly, nematode and vertebrate. In contrast to the neural-specific *fru* gene, *dsx* is involved in sex determination of non-neural as well as neural tissues and *dsx* gene transcripts are translated in both sexes yielding sexually dimorphic protein products, i.e., the male specific protein DsxM and female specific protein DsxF (Shirangi et al., 2006; Kimura et al., 2008: Robinett et al., 2010; Yamamoto and Koganezawa, 2013; Pan et al, 2014; Rideout et al., 2010; Sanders and Arbeitman, 2008).

When *UAS-sbb RNAi* was expressed in the *dsx*-expressing neurons by dsx^{GAL4} in the presence and absence of *UAS-Dcr2*, copulation was completely abolished (Figure 11). This result unequivocally showed that *sbb* function is required in the *dsx*-expressing neurons for normal copulation behavior. The intersectional experiments with neural-specific *elav-GAL80* and glial-specific *Repo-GAL80* demonstrated, respectively, that *sbb*

expression in non-neural and glial *dsx*-expressing neurons has little contribution to copulation. Rather, *sbb* expression in *dsx*-positive neurons seems to be critical for regulation of copulation.

GABAergic and/or serotonergic *dsx*-expressing neurons contribute to the copulation control

dsx-pC1 (including the *fru* positive subset, the P1 cluster) neurons play a pivotal role as the decision-making center for male sexual behavior (Kimura et al., 2008; Kohatsu et al., 2011). Both *dsx*-pC1 and *dsx*-pC2l clusters are involved in following and wing extension actions, which represent the early phase of courtship behavior (Yamamoto et al., 1997). However, only the *dsx*-pC2l cluster has a function to induce the late phase courtship behavior, i.e., licking and attempted copulation (Kohatsu and Yamamoto, 2015). Thus, *dsx*-pC1 and *dsx*-pC2l neurons cooperatively initiate the early and late phase of courtship behavior as a central node. The dsx-positive clusters in lower rung of the CNS include *dsx*-SN, *dsx*-TN1, *dsx*-TN2 and *dsx*-abdominal neurons may serve as sensory or motor centers for controlling mating behavior.

Which subset of *dsx*-expressing neurons is involved in the control of copulation? When *UAS-sbb RNAi* expression *via dsx-GAL4* was suppressed specifically in the thorax by *tsh-GAL80*, the male flies still failed to copulate (Figure 17). The thoracic ganglia harbor the *dsx*-expressing TN1 neurons implicated to function in male-specific courtship songs (Rideout et al., 2007, Clyne et al., 2008). The above result indicates that *sbb* expression in the *dsx*-TN1 neurons is not important for the execution of copulation. Neither *sbb* expression in brain neurons is important for normal copulation as

demonstrated by the experiment with brain-specific *Otd-FLP* (Figure 18). Therefore, the remaining three *dsx*-positive clusters, *dsx*-SN, *dsx*-TN2 and abdominal neurons are candidates for the sites where *sbb* acts for copulation regulation.

My results with transmitter-specific GAL4 drivers strongly suggested that a subset of GABAergic and/or serotoninergic *dsx* neurons in the abdominal ganglion are critical for *sbb*-dependent copulation (Figure 22). GABA is a major inhibitory neurotransmitter in *Drosophila* and other insects and distributed in large numbers of neurons (Sattelle et al., 1991, Enell et al., 2007). Some neurons in the male abdominal ganglion control copulation duration and reproductive fluid transfer (Acebes et al., 2004, Lee et al., 2001). The copulation duration are known to be regulated by 8 GABAergic *dsx*-singly positive interneurons in the abdominal ganglion (Crickmore and Vosshall, 2013). Serotoninergic *fru*-positive neurons in the abdominal ganglion have a role in sperm-pump function, defects in which result in prolonged copulation (Lee et al., 2001). Massive activation of serotoninergic neurons by the temperature-sensitive cation channel, dTRPA1, was shown to inhibit locomotion, feeding and mating in *Drosophila* (Pooryasin and Fiala, 2015). Strikingly, when 5-HT neurons were activated by dTRPA1 in males flies, they did not reduce wing extension frequency but they showed significantly less copulation attempts and lower copulation success (Pooryasin and Fiala, 2015). These results showed that GABAergic and serotoninergic neurons play key roles in the late phase of courtship activity. The present result is consistent with, and extend, these earlier findings by demonstrating that *sbb* is required for the development of GABAergic and serotonergic neurons in the abdominal ganglion that are critically involved in copulation in adult males. This study has opened up a valuable opportunity for future studies in which the

molecular mechanisms underlying instinct can be investigated in the context of developmental orchestration of neural circuit formation dedicated to a specific behavior.

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FIGURE LEGENDS

Figure 1: The genomic region around the *sbb* locus The genetic complementation tests were performed using chromosomal deficiencies. The red bars indicate the deleted segments in respecitive deficiencies. Blue dots indicate the deficiencies that failed to complement the *plt* phenotype, and red dots indicate the deficiencies that complemented *plt*.

Figure 2: **The genetic complementation test for the *plt* short wing vein phenotype (**A) The short wing vein phenotype of a *plt* mutant and normal wing vein of a wild type fly. (B) The frequencies at which the flies showed the short wing vein phenotype in the genetic complementation tests. The short wing vein phenotype was not observed flies carrying a mutant copy of *plt* in *trans* to the homologous chromosome of wild type (+), *Df(2R)BSC483, Df(2R)Excel7153, Df(2R)BSC335* or *Df(2R)BSC339*. The short wing vein phenotype was observed in almost all *plt* heterozygotes that carried, in *trans* to *plt*, *Df(2R)BSC334, Df(2R)ED3683,* $sbb^{BG01610}$, sbb^{6} or sbb^{k00702}(red bars).

Figure 3: **The genetic complementation test of the *plt* copulation phenotype**

The mating success within a 60-minute observation period in the genetic complementation tests. A normal level of mating success was observed in flies carrying a mutant copy of *plt* in *trans* to the chromosome that was wild type (+), *Df(2R)BSC483, Df(2R)Excel7153, Df(2R)BSC335* or *Df(2R)BSC339* (blue bars). Copulation was not observed in flies carrying a mutant copy of *plt* in *trans* to the chromosome that was

Df(2R)BSC334, Df(2R)ED3683, $sbb^{BG01610}$, sbb^{6} or *sbb^{k00702}* The numbers of flies examined are shown on the top of bars.

Figure 4: **Courtship activities in flies used for the genetic complementation test** The courtship index of flies carrying a mutant copy of *plt* in *trans* to the chromosome that was wild type (+), deficiencies or *sbb* alleles within a 10-minute observation period. The numbers on the top of bars show the number of tested flies.

Figure 5: **Exon-intron organization of *sbb* and the *P* element inserted point in the *plt* mutant** (A) A *P* element (shown by a triangle) in the *plt* mutant was inserted in the *sbb* locus. The multiple *sbb* transcripts encoded by the locus are shown. Gray box, untranslated region; yellow box, protein coding sequence; black line, intron.
(B) *Drosophila* genomic DNA sequence around the *P*-element insertion site in *plt.* The *P* element locates 31-bp downstream of the transcription start site of *sbb*. Blue letters indicate a part of the *sbb* transcript. Red letters indicate the 8-bp target sequence of the *P* element.

Figure 6: The short wing vein phenotype of *plt* was reproduced by the *sbb* knockdown at the late larval stage The wing vein phenotype of emerged flies of *hs-GAL4, UAS-sbb RNAi* (red bars) and negative controls. A heat shock at 37°C was applied for 1 hour at the larval or pupal stage. The numbers on the top of bars show the number of tested flies.

Figure 7: **The copulation phenotype of *plt* was reproduced by the *sbb* knockdown at the late larval stage** The mating success within a 60 minute observation period in *hs-GAL4, UAS-sbb RNAi* (blue bars) and in negative controls. *hs-GAL4/+* and *UAS-sbb RNAi/+* flies were controls (grey bars). A heat shock was applied at the larval or pupal stage. Mating success was decreased when the expression of *UAS-sbb RNAi* was induced by applying a heat shock at the larval stage, but not when the heat shock was applied at the pupal stage. The numbers on the top of bars show the number of tested flies. * indicates $p < 0.05$ in the Chi-square test.

Figure 8: ***sbb* knockdown in neurons led the copulation defect** (A) The mating success within a 60 minute observation period in $elav^{C155}$*, UAS-sbb RNAi* with and without *UAS-Dcr2* (blue bars) and in negative controls. $elav^{C155}$/+and *UAS-sbb RNAi VDRC/+* flies were controls as indicated by grey bars. The mating successes of $elav^{C155}$*, UAS-sbb RNAi* with or without *UAS-Dcr2* were lower than those of negative controls. The numbers on the top of bars show the number of tested flies. (B) The courtship index within a 10 minute observation period in $elav^{C155}$*, UAS-sbb RNAi* with or without *UAS-Dcr2* (green bars) and in negative controls (grey bars). There was no significant difference among the flies of indicated genotypes. The numbers on the top of bars show the number of tested flies. ** indicates $p < 0.01$, *** indicates $p < 0.001$ in the Chi-square test.

Figure 9: ***sbb* knockdown in fru^{GAL4}-expressing cells led to a moderate copulation defect (**A) The mating success within a 60 minute observation period in fru^{GAL4}*, UAS-sbb RNAi* in the presence and absence of *UAS-Dcr2* (blue bars) and in negative controls.

fru^{GAL4}/+ and *UAS-sbb RNAi/+* flies were controls (grey bars). The test flies showed a moderate reduction in mating success. (B) The courtship index within a 10 minute observation period in *fru*^{GAL4}*, UAS-sbb RNAi* (green bars) and in negative controls (grey bars). There was no significant difference among the flies of indicated genotypes. The numbers on the top of bars show the number of tested flies. ** indicates $p < 0.01$, *** indicates $p < 0.001$ in the Chi-square test.

Figure 10: ***sbb* knockdown in *fru*^{NP21}-expressing cells led to the copulation defect (**A) The mating success within a 60 minute observation period in *fru*^{NP21}*, UAS-sbb RNAi in the* presence and absence of *UAS-Dcr2* (blue bars) and in negative controls. *fru*^{NP21}/+ and *UAS-sbb RNAi/+* flies were controls (grey bars). The mating success was significantly decreased in the test flies. (B) The courtship index within a 10 minute observation period in *fru*^{NP21}*, UAS-sbb RNAi* (green bars) and in negative controls (grey bars). There was no significant difference among the flies of indicated genotypes. The numbers on the top of bars show the number of tested flies.

** indicates $p < 0.01$, *** indicates $p < 0.001$ in the Chi-square test.

Figure 11: *sbb* knockdown in *dsx*-expressing cells led to the strong copulation defect (A) Expression of *dsx*^{GAL4} in neurons and associated projections in the 8-day adult male brain (top) and ventral nerve cord (VNC, bottom). The pC1 and pC2l clusters and SN neurons are seen in the brain (arrow). Adult males have *dsx*^{GAL4}expression in the male-specific TN1 and TN2 neuronal clusters in the thoracic ganglia and a large number abdominal neurons in the abdominal ganglion. (B) The mating success within a 60

minute observation period in *dsx*^{GAL4}*, UAS-sbb RNAi* in the presence and absence of *UAS-Dcr2* and in negative controls. *dsx*^{GAL4}/+ with *UAS-Dcr2*/+ and *UAS-sbb RNAi*/+ flies were controls (grey bars). Copulation was completely inhibited in the test flies. (C) The courtship index within a 10 minute observation period in *dsx*^{GAL4}*, UAS-sbb RNAi* in the presence and absence of *UAS-Dcr2* (green bars) and in negative controls (grey bars). There was no significant difference among the flies of indicated genotypes. The numbers on the top of bars show the number of tested flies.

*** indicates $p < 0.001$ in the Chi-square test. The Mann-Whitney's U-test statistical test was used for the courtship index. Scale bar: 50 μm.

Figure 12: *sbb* knockdown in the *dsx* and *fru* double positive cells hardly affected copulation (A) A schematic of the intersectional genetic strategy to label and manipulate *dsx* and *fru* double positive neurons. (B) The mating success within a 60 minute observation period in flies carrying *dsx*^{GAL4}*, fru*^{FLP}*, UAS-sbb RNAi* and *tubP>GAL80>* in the presence of *UAS-Dcr2* (blue bar, *dsx* and *fru* double positive neurons) and in negative controls. *dsx*^{GAL4}*/ UAS-sbb RNAi* with *UAS-Dcr2* and *UAS-sbb RNAi/ fru*^{FLP} flies were controls (grey bars). The mating success was high in test flies. (C) The courtship index within a 10 minute observation period in flies with the genotype of *dsx*^{GAL4}*, UAS-sbb RNAi, fru*^{FLP}*, tubP >GAL80>*in the presence of *UAS-Dcr2* (green bar) and in negative controls (grey bars). There was no significant difference among the flies with indicated genotypes. The numbers on the top of bars show the number of tested flies.

*** indicates $p < 0.001$ in the Chi-square test. The Mann-Whitney's U-test statistical test was used for the courtship index.

Figure 13: *sbb* knockdown in *fru* single positive cells did not affect copulation

(A) A schematic of the intersectional genetic strategy to label and manipulate *fru* single positive neurons. (B) The mating success within a 60 minute observation period in flies with the genotype of fru^{GAL4}, dsx^{FLP}, *UAS-sbb RNAi* with *tubP>stop>GAL80* (blue bar, *fru* single positive neurons) and in negative controls. Flies of the genotype: fru^{GAL4}/ *UAS-sbb RNAi* with *tubP>stop>GAL80* and those: *UAS-sbb RNAi/* fru^{GAL4} served as controls (grey bars). All test flies copulated. (C) The courtship index within a 10 minute observation period in flies with the genotype of fru^{GAL4}, dsx^{FLP}, *UAS-sbb RNAi* with *tubP>stop>GAL80* (green bar) and in negative controls (grey bars). There was no significant difference among the flies of indicated genotypes. The numbers on the top of bars show the number of tested flies.

* indicates $p < 0.05$ in the Chi-square test. The Mann-Whitney's U-test statistical test was used for the courtship index.

Figure 14: *sbb* knockdown in *dsx* single positive cells strongly affected copulation A) A schematic of the intersectional genetic strategy to label and manipulate *dsx* single positive neurons. (B) The mating success within a 60 minute observation period in flies with the genotype of dsx^{GAL4}, fru^{LexA}, *LexAop-GAL80, UAS-sbb RNAi* with *UAS-Dcr2* (*dsx* single positive neurons) and in negative controls. Flies of the genotype: *UAS-sbb RNAi* with fru^{LexA}, *LexAop-GAL80* and those: *UAS-sbb RNAi/* dsx^{GAL4} with *UAS-Dcr2* were controls (grey bars). All test flies did not copulate. (C) The courtship index within a 10 minute observation period in flies with the genotype of dsx^{GAL4}, fru^{LexA}, *LexAop-GAL80, UAS-sbb RNAi* with *UAS-Dcr2* (green bar) and in negative controls (grey bars). There was no significant difference among the flies of the indicated genotypes. The numbers on the top of bars show the number of tested flies.

*** indicates $p < 0.001$ in the Chi-square test. The Mann-Whitney's U-test statistical test was used for the courtship index.

Figure 15: ***sbb*** **knockdown in non-neural** ***dsx*****-expressing cells did not affect copulation** (A) A schematic illustrating the portions in which GAL4 is active (glia, red) or inactive (neurons, black) in the brain (top) and VNC (bottom). (B) The mating success within a 60 minute observation period in flies with the genotype of dsx^{GAL4}, *UAS-sbb RNAi* with *elav-GAL80* and in negative controls. Flies of the genotype: dsx^{GAL4} with *UAS-sbb RNAi* and those: *UAS-sbb RNAi/ elav-GAL80* were controls (grey bars). All of experimental flies copulated (blue bar). (C) The courtship index within a 10 minute observation period in flies with the genotype of dsx^{GAL4}, *UAS-sbb RNAi* with *elav-GAL80* (green bar) and negative controls (grey bars). There was no significant difference among the flies of indicated genotypes. The numbers on the top of bars show the number of tested flies.

*** indicates $p < 0.001$ in the Chi-square test. The Mann-Whitney's U-test statistical test was used for the courtship index.

Figure 16: ***sbb*** **knockdown in the non-glial** ***dsx*****-expressing cells led to the copulation defect** (A) A schematic illustrating the portions in which GAL4 is active (neurons, red region) or inactive (glia, black) in the brain (top) and VNC (bottom). (B) The mating success within a 60 minute observation period in flies of the genotype of dsx^{GAL4}, *UAS-sbb RNAi* with *Repo-GAL80* and in negative controls. Flies of the genotype: dsx^{GAL4} with *UAS-sbb RNAi* and those: *UAS-sbb RNAi/+* were controls (grey bars). The test flies did not copulate. (C) The courtship index within a 10 minute of observation period in flies of

the genotype *dsx^{GAL4}, UAS-sbb RNAi* with *Repo-GAL80* (green bar) and in negative controls (grey bars). There was no significant difference among the flies of indicated genotypes. The numbers on the top of bars show the number of tested flies.

*** indicates $p < 0.001$ in the Chi-square test. The Mann-Whitney's U-test statistical test was used for the courtship index.

Figure 17: *sbb* expression in brain *dsx*-expressing neurons is dispensable for normal copulation (A) The CNS of a 8-day adult male expression of *dsx-GAL4* was inhibited in the thorax by *teashirt-GAL80* (*tsh-GAL80*). The pC1 and pC2l clusters and SN neurons in the brain the TN2 cluster in the thoracic ganglion and abdominal neurons are positive for GAL4 (arrow). The TN1 cluster in the thoracic ganglion is negative for GAL4. (B) The mating success within a 60 minute observation period in flies of the genotype of *dsx^{GAL4}, UAS-sbb RNAi* with *tsh-GAL80* and in negative controls. Flies of the genotype: *dsx^{GAL4}* with *UAS-sbb RNAi* and those: *UAS-sbb RNAi/+* were controls (grey bars). The mating success was blocked in the experimental flies. (C) The courtship index within a 10 minute observation period in flies of the genotype of *dsx^{GAL4}, UAS-sbb RNAi* with *tsh-GAL80* and in negative controls. There was no significant difference among the flies with indicated genotypes . The numbers on the top of bars show the number of tested flies.

*** indicates $p < 0.001$ in the Chi-square test. The Mann-Whitney's U-test statistical test was used for the courtship index. Scale bar: 50 μm.

Figure 18: *sbb* expression in *dsx* positive TN1 neurons are dispensable for controlling the copulation (A) Expression of *dsx^{GAL4}* in the presence of brain specific *Otd-FLP* and *tubP>GAL80>* in the 8-day adult male brain (top) and ventral nerve cord (VNC, bottom). SN, TN1, TN2 and abdominal neurons are negative for GAL4

expression. The *dsx*-expressing neurons in the brain including the pC1, pC2l clusters are positive for GAL4 expression. **(**B) The mating success within a 60 minute observation period in flies with the genotype of dsx^{GAL4}*, Otd-FLP, UAS-sbb RNAi, UAS-Dcr2* with *tubP>GAL80>* (blue bar) and in negative controls (grey bars). The mating success was high in test flies. (C) The courtship index within a 10 minute observation period in flies with the genotype of dsx^{GAL4}*, Otd-FLP, UAS-sbb RNAi, UAS-Dcr2* with *tubP>GAL80>* (green bar) and in negative controls (grey bars). There was no significant difference among the flies with indicated genotypes. The numbers on the top of bars show the number of tested flies.

*** indicates $p < 0.001$ in the Chi-square test. The Mann-Whitney's U-test statistical test was used for the courtship index. Scale bar: 50 μm.

Figure 19: ***sbb*** **expression in** ***dsx*****-expressing SN, TN2 neurons and abdominal neurons are critical for normal copulation (**A) Expression of dsx^{GAL4} in the presence of brain specific *Otd-FLP* and *tubP>stop>GAL80* in the 8-day adult male brain (top) and ventral nerve cord (VNC, bottom). SN, TN1, TN2 and abdominal neurons are positive for GAL4 expression. The *dsx*-expressing neurons in the brain including the pC1, pC2l clusters are negative for GAL4 expression. **(**B) The mating success within a 60 minute observation period in flies of the genotype of dsx^{GAL4}*, Otd-FLP, UAS-sbb RNAi,* with *tubP >stop>GAL80* and in negative controls (grey bars). The test flies did not copulate. (C) The courtship index within a 10 minute observation period in flies of the genotype of dsx^{GAL4}*, Otd-FLP, UAS-sbb RNAi* with *tubP >stop>GAL80* (green bar) and in negative controls (grey bars). There was no significant difference among the flies of indicated genotypes. The numbers on the top of bars show the number of tested flies.

*** indicates $p < 0.001$ in the Chi-square test. The Mann-Whitney's U-test statistical test was used for the courtship index. Scale bar: 50 μm.

Figure 20: *sbb* expression in non-cholinergic *dsx*-expressing neurons are critical for normal copulation (A) The CNS of an 8-d adult male in which expression of *dsx-GAL4* was excluded from cholinergic neurons by *Cha-GAL80*. The pC1 and pC2l clusters and TN1 neurons are negative for GAL4. The SN, a subset of TN2 and abdominal neurons are positive for GAL4 (arrow). (B) The mating success within a 60 minute observation period in flies with the genotype of dsx^{GAL4}, *UAS-sbb RNAi* with *Cha-GAL80* and in negative controls. Flies carrying dsx^{GAL4} and *UAS-sbb RNAi* and those carrying *Cha-GAL80/+* were controls (grey bar). Copulation was blocked in the experimental flies. (C) The courtship index within a 10 minute observation period in flies with the genotype of dsx^{GAL4}, *UAS-sbb RNAi* with *Cha-GAL80* (green bar) and in negative controls (grey bars). There was no significant difference among the flies of indicated genotypes. The numbers on the top of bars show the number of tested flies.

*** indicates $p < 0.001$ in the Chi-square test. The Mann-Whitney's U-test statistical test was used for the courtship index. Scale bar: 50 μm.

Figure 21: *sbb* expressed in the *dsx* and *poxn* double positive neurons partially contribute to copulation (A) A schematic of the intersectional genetic strategy to label and manipulate *dsx* and *ppk* or *dsx* and *poxn* double positive neurons. (B) The mating success within a 60 minute observation period in flies with the genotype of dsx^{FLP}, *UAS-sbb RNAi, tubP>GAL80>* with *ppk-GAL4* or *poxn-GAL4* (blue bars) and in a negative control. Flies having dsx^{FLP}, *UAS-sbb RNAi, tubP>GAL80>* without GAL4 drivers were controls (grey bar). The mating success was high in test flies with *ppk-GAL4*, whereas

test flies with *poxn-GAL4* exhibited reduced mating success. (C) The courtship index within a 10 minute observation period in flies of the genotype of *dsx^{FLP}, UAS-sbb RNAi, tubP>GAL80>* with *ppk-GAL4* or *poxn-GAL4* (green bars) and in negative controls (grey bar). There was no significant difference among the flies of indicated genotypes. The numbers on the top of bars show the number of tested flies.

* indicates $p < 0.05$ in the Chi-square test. The Mann-Whitney's U-test statistical test was used for the courtship index.

Figure 22: *sbb* knockdown in GABAergic and serotonergic *dsx*-expressing neurons impaired copulation (A) A schematic of the intersectional genetic strategy to label and manipulate *dsx* neurons that are also positive for different GAL4 drivers specified by neurotransmitters. (B) The mating success within a 60 minute observation period in flies of the genotype of *dsx^{FLP}, UAS-sbb RNAi, tubP>GAL80>* with *Cha-GAL4*, *TH-GAL4*, *GAD-GAL4, Vglut-GAL4, Tdc2-GAL4* or *Trh-GAL4* (blue bars) and in a negative control (CS). Flies having *dsx^{FLP}, UAS-sbb RNAi, tubP>GAL80>* without GAL4 drivers were controls (grey bar). (C) The courtship index within a 10 minute observation period in flies of the genotype of *dsx^{FLP}, UAS-sbb RNAi, tubP>GAL80>* with *Cha-GAL4*, *TH-GAL4*, *GAD-GAL4, Vglut-GAL4, Tdc2-GAL4* or *Trh-GAL4* (green bars) and in a negative control (grey bar). There was no significant difference among the flies of indicated genotypes.

*indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ in the Chi-square test. The Mann-Whitney's U-test statistical test was used for the courtship index.

Figure 23: GABAergic and serotonergic *dsx*-expressing neurons in the abdominal ganglion that potentially contribute to *sbb*-dependent copulation control (A) Putative glutamatergic *dsx* neurons in the CNS of an 8-d adult male revealed by using dsx^{FLP}, *Vglut-GAL4* with *UAS>stop>mCD8::GFP* (a membrane-bound GFP reporter). (B) Putative GABAergic *dsx* neurons in the CNS of an 8-d adult male revealed by using dsx^{FLP}, *GAD-GAL4* on the 2nd chromosome with *UAS>stop>mCD8::GFP*. C) Putative GABAergic *dsx* neurons in the CNS of an 8-d adult male revealed by using dsx^{FLP}, *GAD-GAL4* on the 3rd chromosome with *UAS>stop>mCD8::GFP*. D) Putative serotonergic *dsx* neurons in the CNS of an 8-d adult male revealed by using dsx^{FLP}, *Trh-GAL4* on the 2nd chromosome with *UAS>stop>mCD8::GFP*. Scale bar: 50 μm.

Figure 1

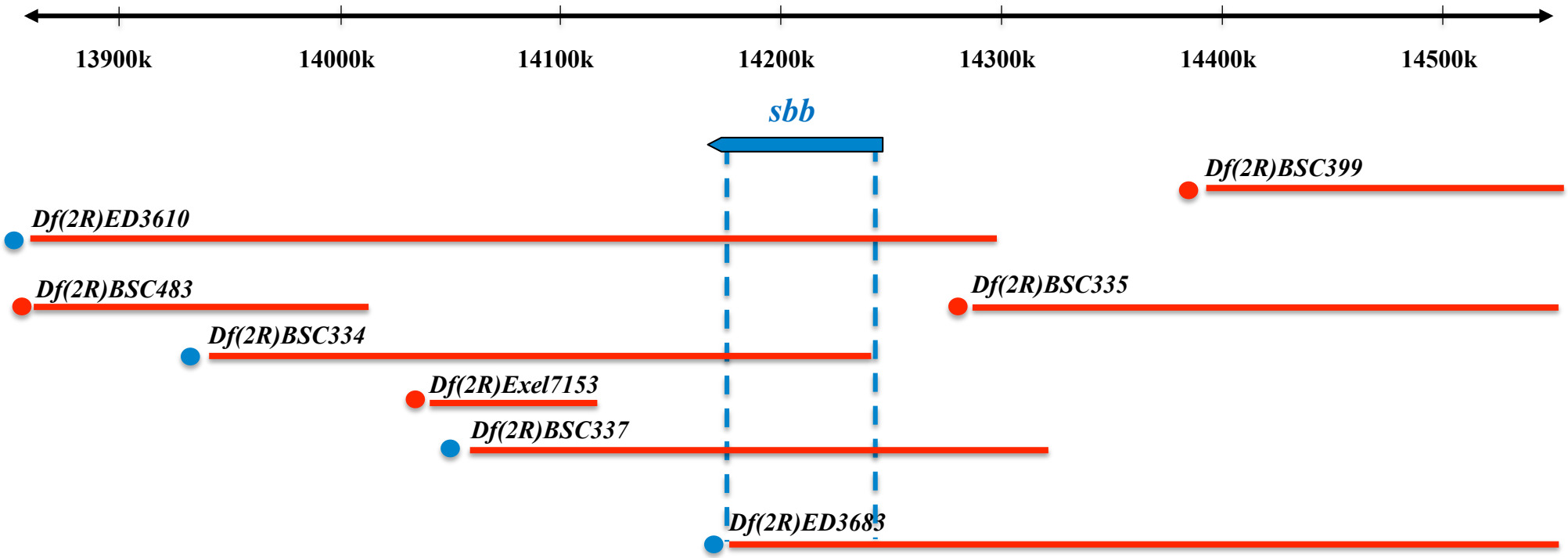

13900k
14000k
14100k
14200k
14300k
14400k
14500k
sbb
Df(2R)BSC399
Df(2R)ED3610
Df(2R)BSC483
Df(2R)BSC335
Df(2R)BSC334
Df(2R)Exel7153
Df(2R)BSC337
Df(2R)ED3683

Figure 2

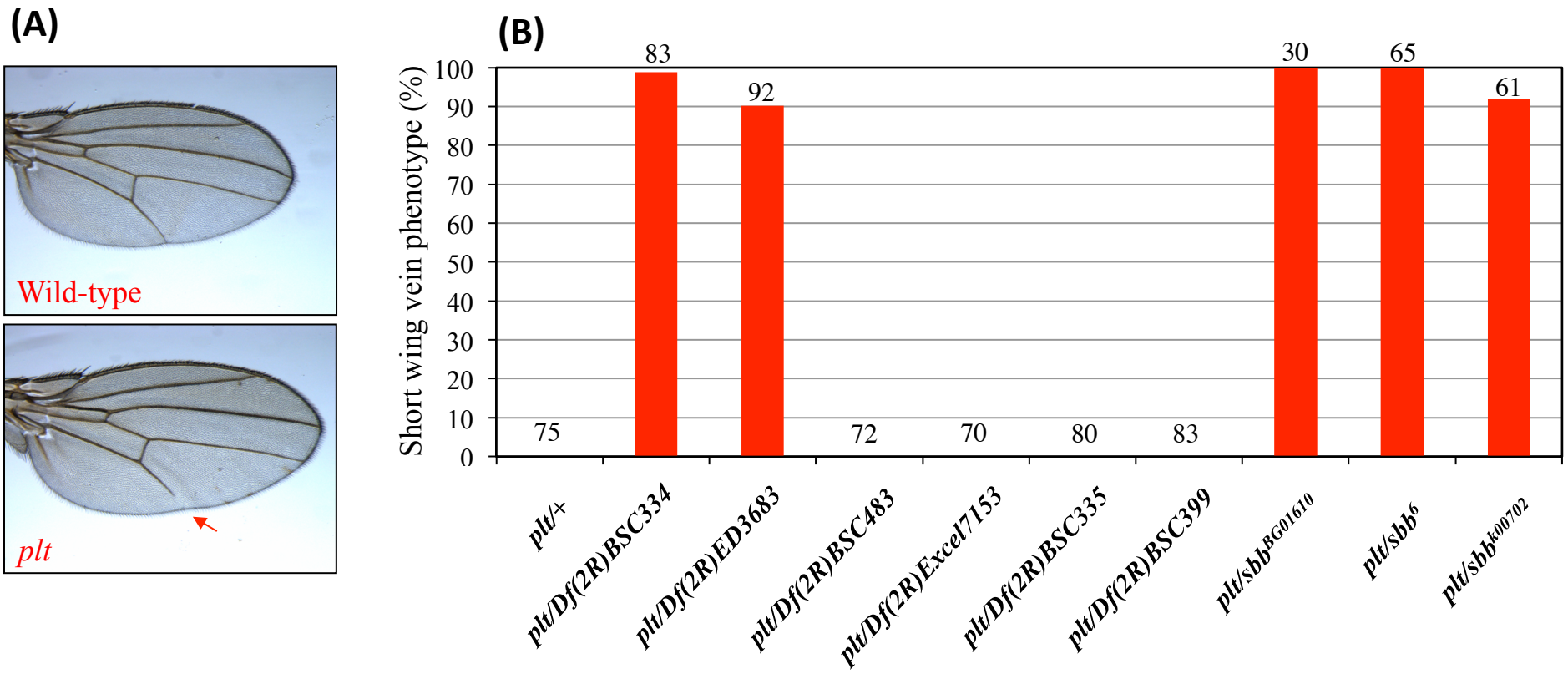

(A)
Wild-type
plt
(B)
Short wing vein phenotype (%)
100
90
80
70
60
50
40
30
20
10
0
75
83
92
72
70
80
83
30
65
61
plt/+
plt/Df(2R)BSC334
plt/Df(2R)ED3683
plt/Df(2R)BSC483
plt/Df(2R)Excel7153
plt/Df(2R)BSC335
plt/Df(2R)BSC399
plt/sbb^BG01610
plt/sbb^6
plt/sbb^k00702

Figure 3

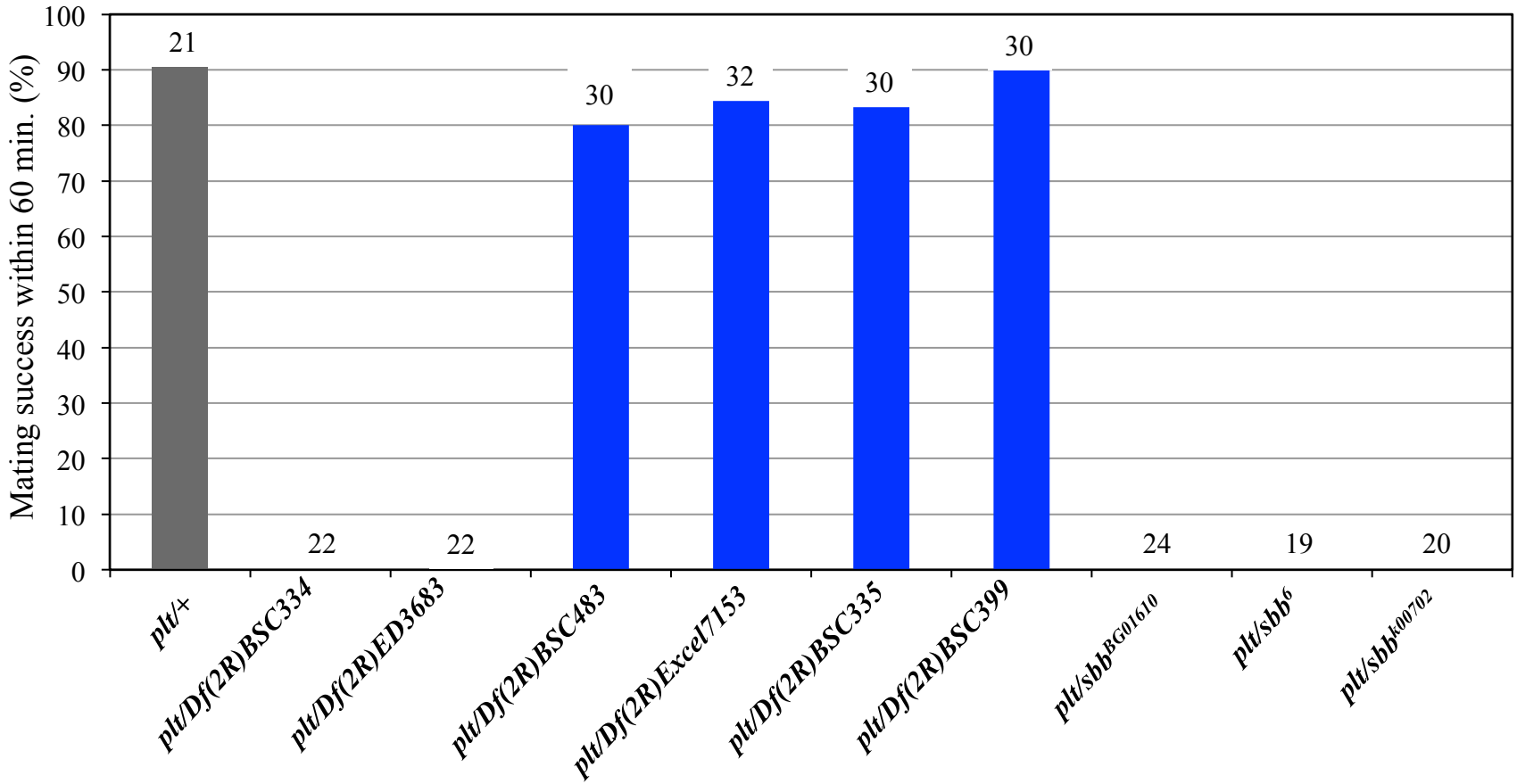

Mating success within 60 min. (%)
100
90
80
70
60
50
40
30
20
10
0
21
22
22
30
32
30
30
24
19
20
plt/+
plt/Df(2R)BSC334
plt/Df(2R)ED3683
plt/Df(2R)BSC483
plt/Df(2R)Exel7153
plt/Df(2R)BSC335
plt/Df(2R)BSC399
plt/sbb^BG01610
plt/sbb^6
plt/sbb^k00702

Figure 4

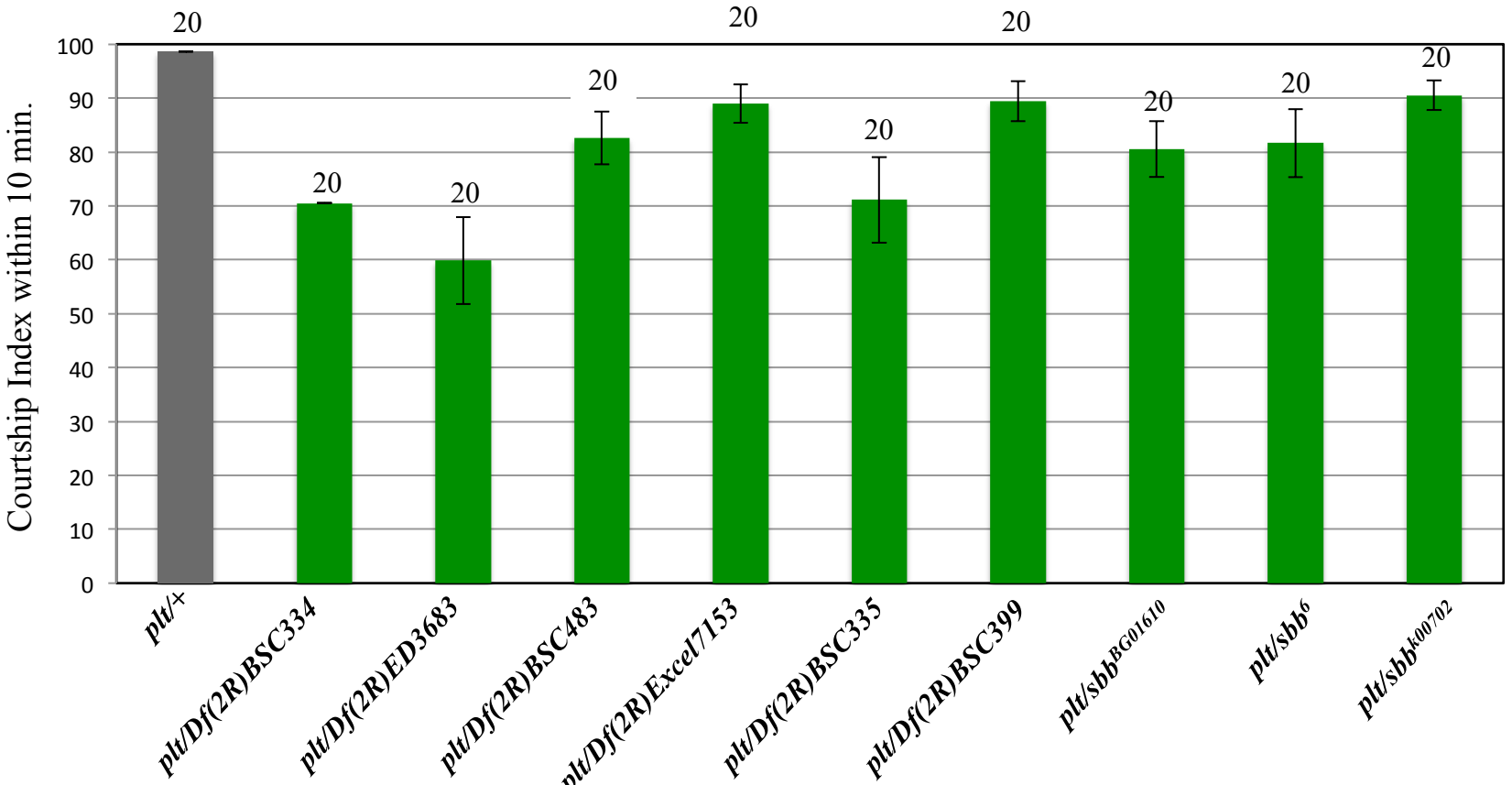
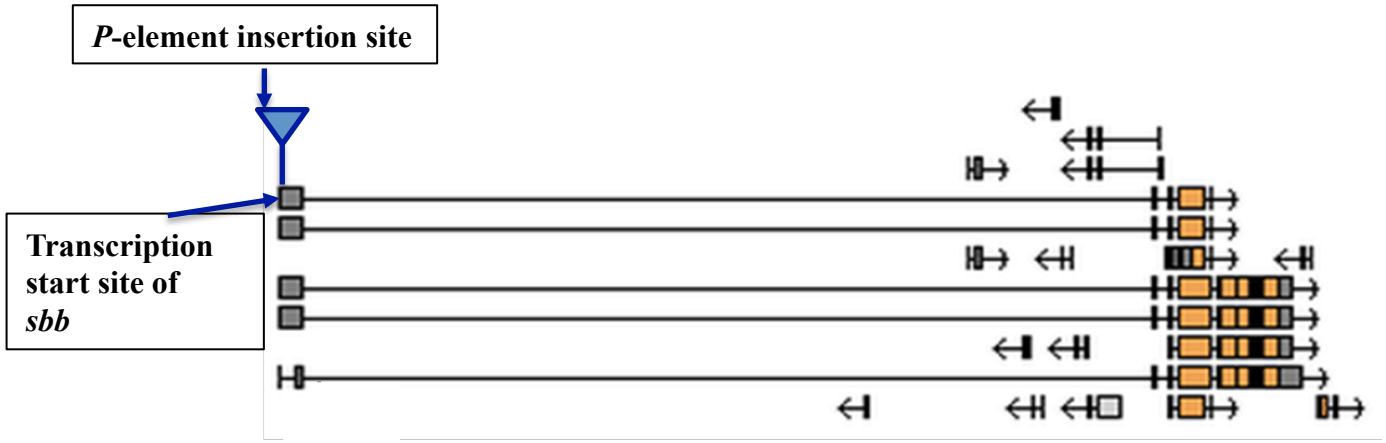

Courtship Index within 10 min.
100
90
80
70
60
50
40
30
20
10
0
20
20
20
20
20
20
20
20
20
plt/+
plt/Df(2R)BSC334
plt/Df(2R)ED3683
plt/Df(2R)BSC483
plt/Df(2R)Excel7153
plt/Df(2R)BSC335
plt/Df(2R)BSC399
plt/sbb^BG01610
plt/sbb^6
plt/sbb^k00702

Figure 5 **(A)**



(B)

P-element insertion site

sbb sequence

GGCTGAGAATATTAACAAGTAAATAATCTTTTTATTAGTAAATTGTAACTTTATTGCTTTTA
AGAGTTTGAAATCGCGATAACTATATCAGAGAAGCATTCGACTAAAAACAAGAATTCTTT
CGGAACCATATAATTACCGAAATGTATTTGTTATTTTTCCCCATATAGCCGACAACCCTGC
GCGTTGCTAGCCAACTCTCGCCTCTCAATTTATGCGCTTTTGTACGCGTTGCTCCTTCGGT
TCGTTCGGACAGCACCGGATAAAACGCTGTCGCCAAACAAAAACATAAAAAACACTGAG
CGGATCAGGTTCTATCTTTAATTAAGAAAAACAACATCCCAAAAACAACAAGCAAGAAG
GACTAAAATCTGTGGAAAAGAAGTAAGCAAAGCTGTTCCCCAGTGTGATTTCCTCATGT
GCATTTAATGTAATTCAACTCGACAACAAAATAATAAGTGTTGTTGGCCTCTCGCTCTCT
GGTCGTACCGCGTATCTGTGTGTGCGTGATTTAGTGTTTCGTCGCGTTTCAAGCAATTAA
CCCAAAGCAACGTAGAAATGCCAAAAACGCATTAAAAATTATTACTCGTAGCTGTCTGT
CATTCACAACAGAAAAAAAAAATGAAAAGAGAAGAGTAACAACAGCAACGAGAAGAA
GGAGGAGAGCGACAATCATACACAAGTGCTATTTGCCGCTACTCATAAAACAAATTACC
CACTTGGGCC

Transcription start site of *sbb*

Figure 6

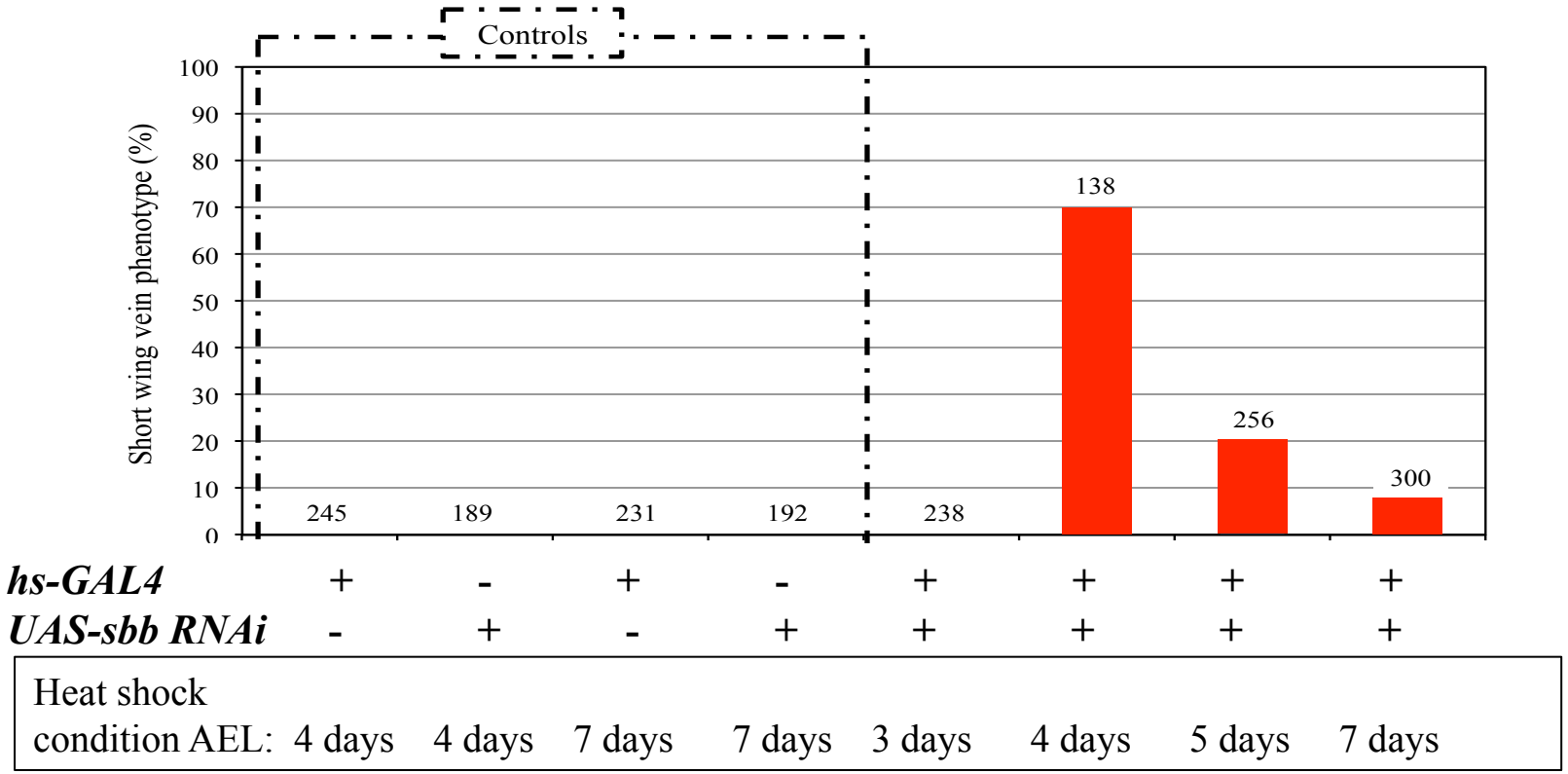

Controls
Short wing vein phenotype (%)
100
90
80
70
60
50
40
30
20
10
0
245
189
231
192
238
138
256
300
hs-GAL4 + - + - + + + +
UAS-sbb RNAi - + - + + + + +
Heat shock
condition AEL: 4 days 4 days 7 days 7 days 3 days 4 days 5 days 7 days

Figure 7

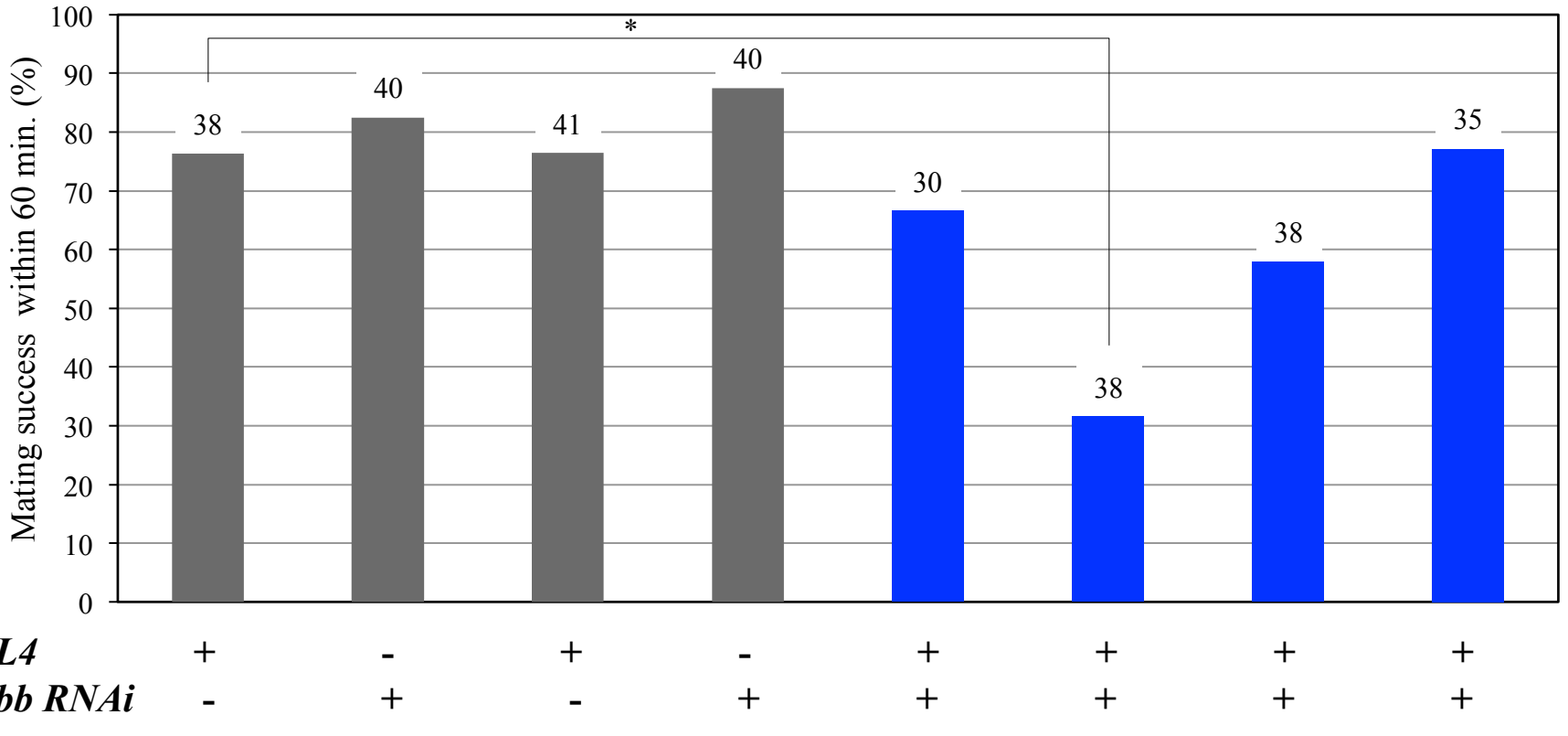

Mating success within 60 min. (%)
100
90
80
70
60
50
40
30
20
10
0
*
38
40
41
40
30
38
35
hs-GAL4 + - + - + + + +
UAS-sbb RNAi - + - + + + + +
Heat shock condition AEL: 4 days 4 days 7 days 7 days 3 days 4 days 5 days 7 days

Figure 8

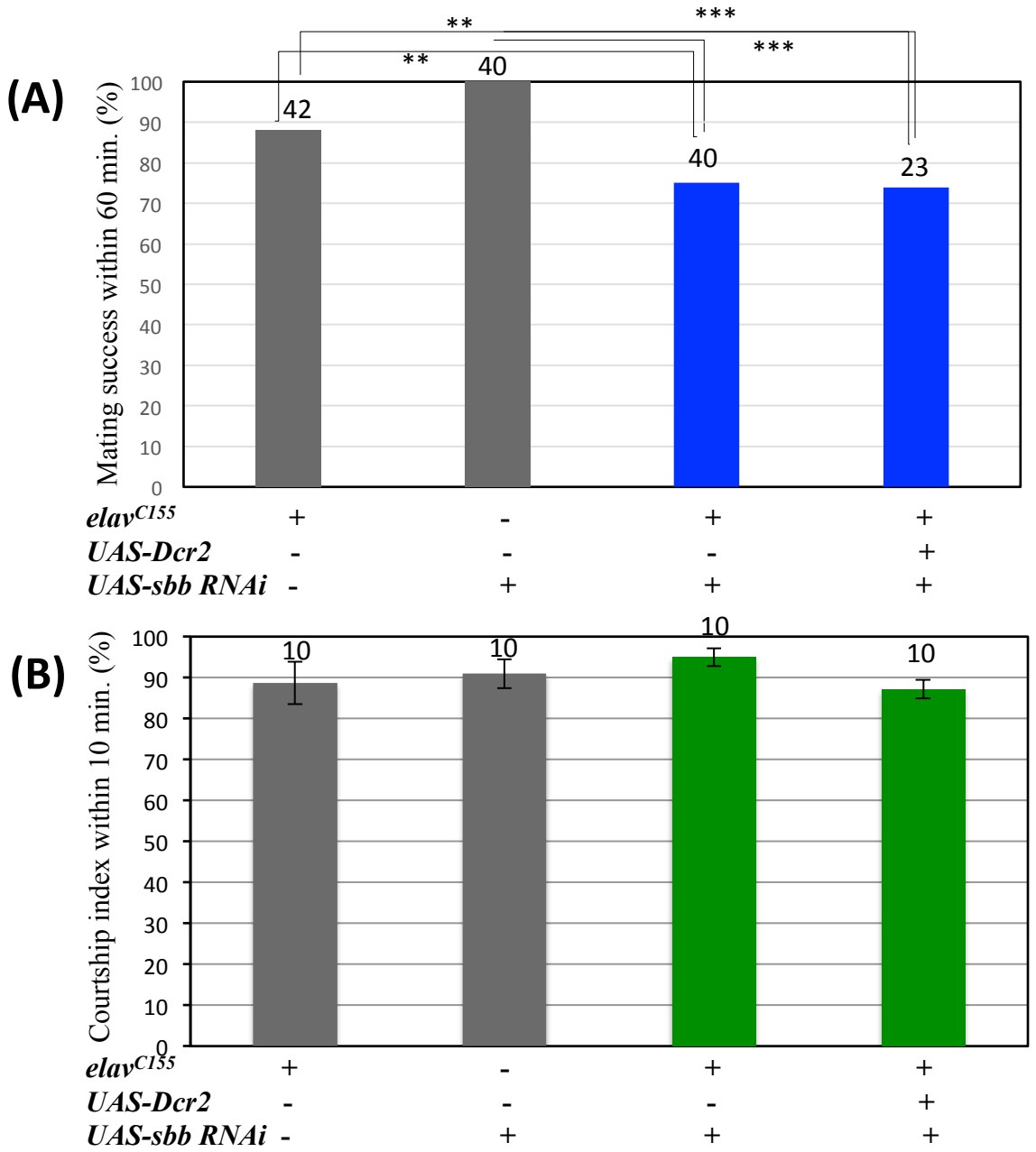

(A)
Mating success within 60 min. (%)
100
90
80
70
60
50
40
30
20
10
0
**
**
***
***
42
40
40
23
elav^C155 + - + +
UAS-Dcr2 - - - +
UAS-sbb RNAi - + + +
(B)
Courtship index within 10 min. (%)
10
10
10
10
elav^C155 + - + +
UAS-Dcr2 - - - +
UAS-sbb RNAi - + + +

Figure 9

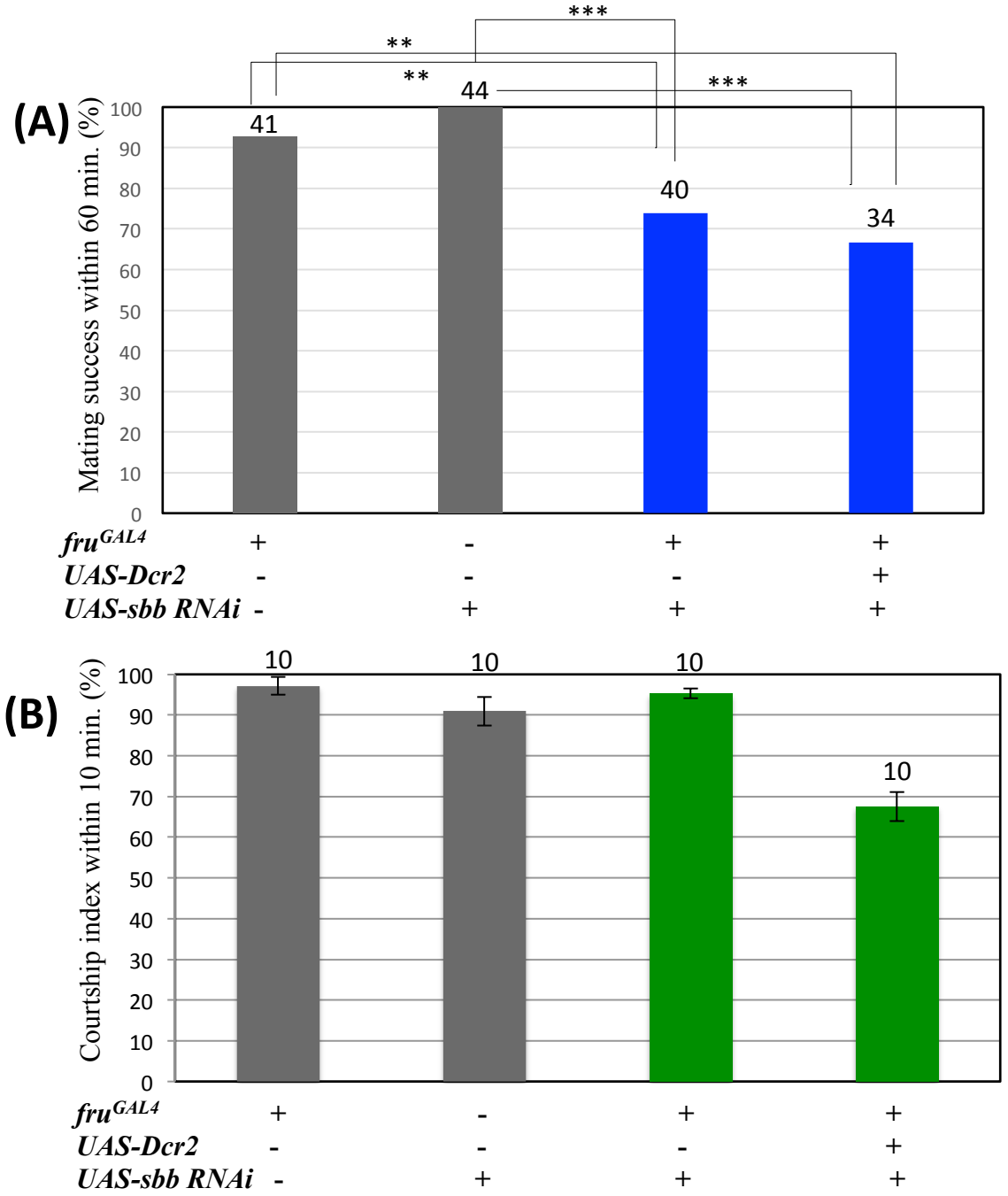

(A)
Mating success within 60 min. (%)
100
90
80
70
60
50
40
30
20
10
0
41
44
40
34
**
**
***
***
fruGAL4 + - + +
UAS-Dcr2 - - - +
UAS-sbb RNAi - + + +
(B)
Courtship index within 10 min. (%)
10
10
10
10
fruGAL4 + - + +
UAS-Dcr2 - - - +
UAS-sbb RNAi - + + +

Figure 10

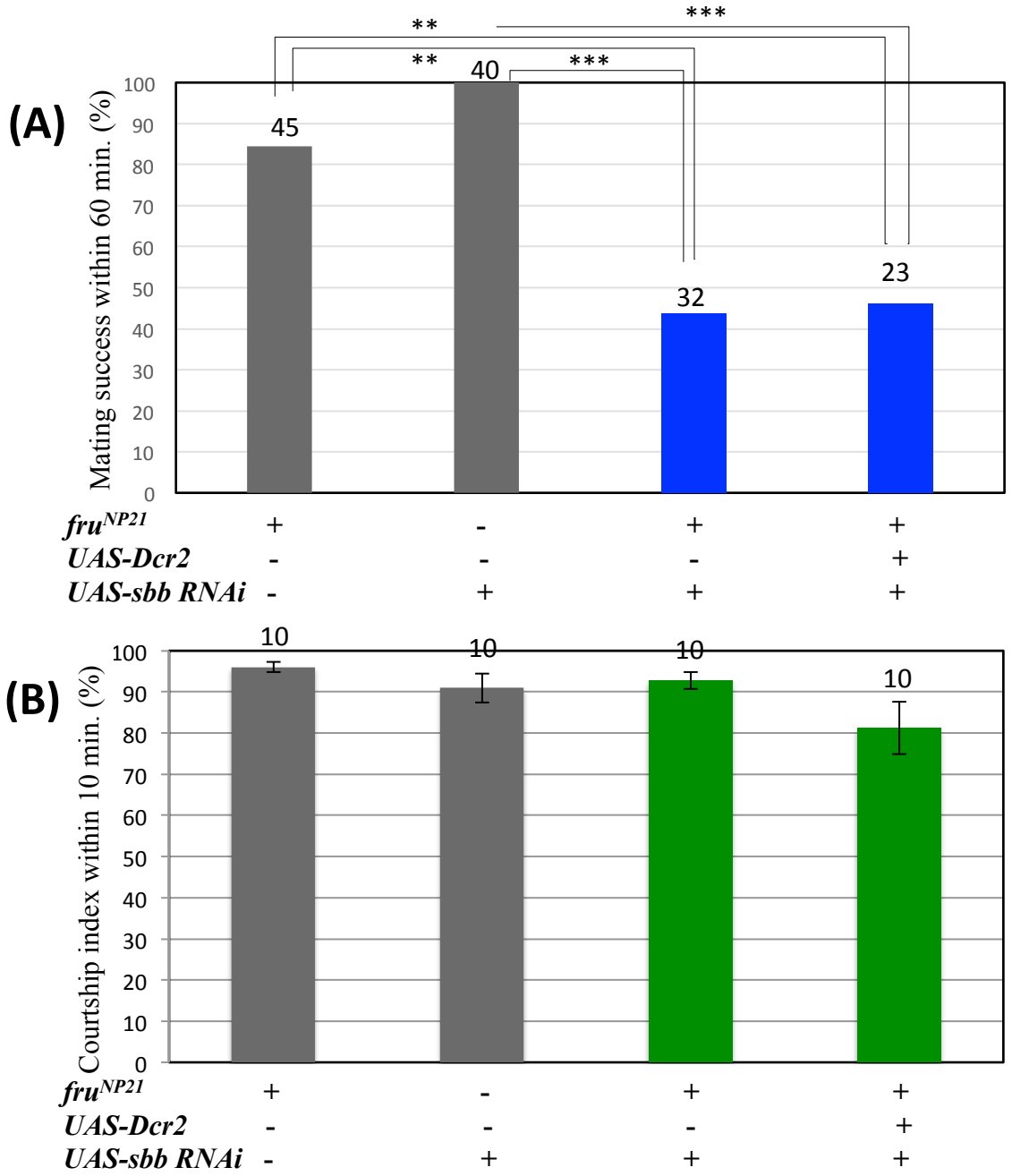
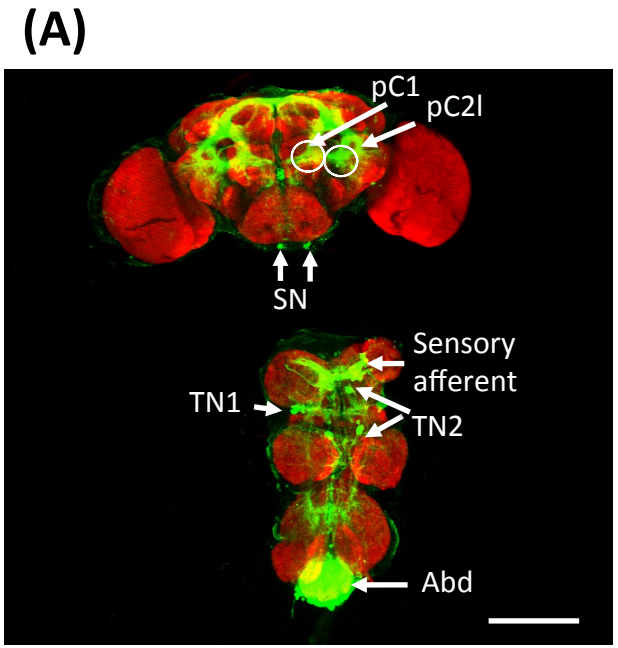
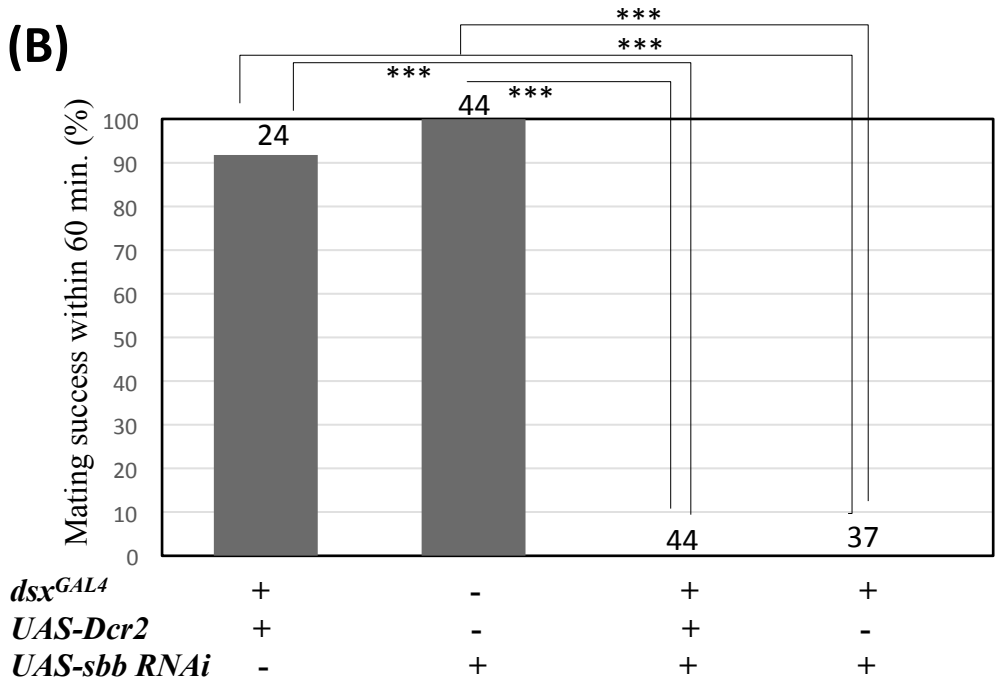

(A)
Mating success within 60 min. (%)
**
**
***
***
45
40
32
23
fruNP21 + - + +
UAS-Dcr2 - - - +
UAS-sbb RNAi - + + +
(B)
Courtship index within 10 min. (%)
10
10
10
10
fruNP21 + - + +
UAS-Dcr2 - - - +
UAS-sbb RNAi - + + +

Figure 11



w; Sp/CyO; dsx^{GAL4} /UAS-mCD8::GFP



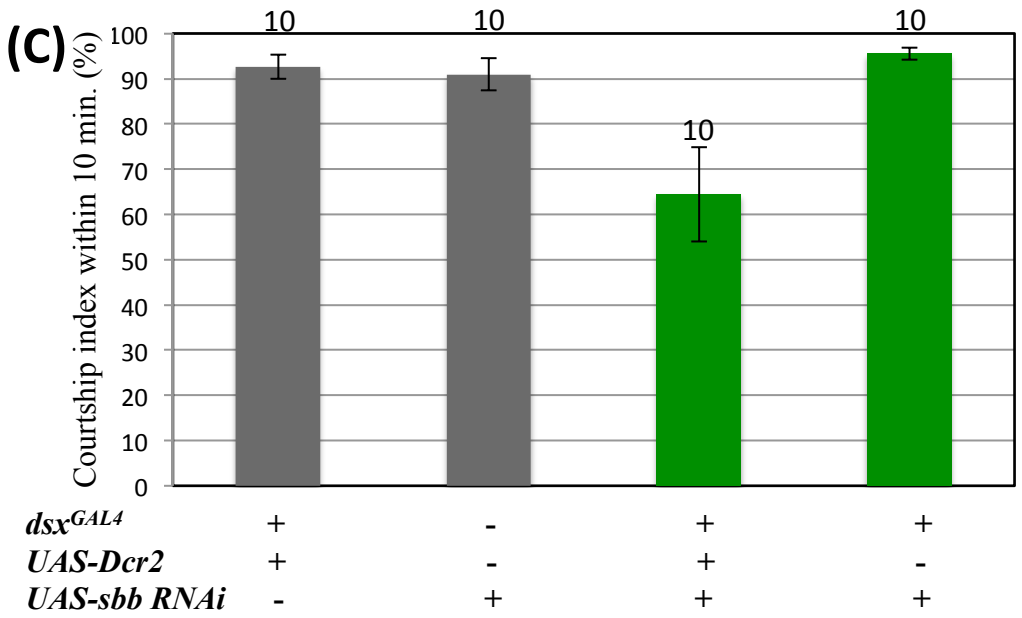


Figure 12

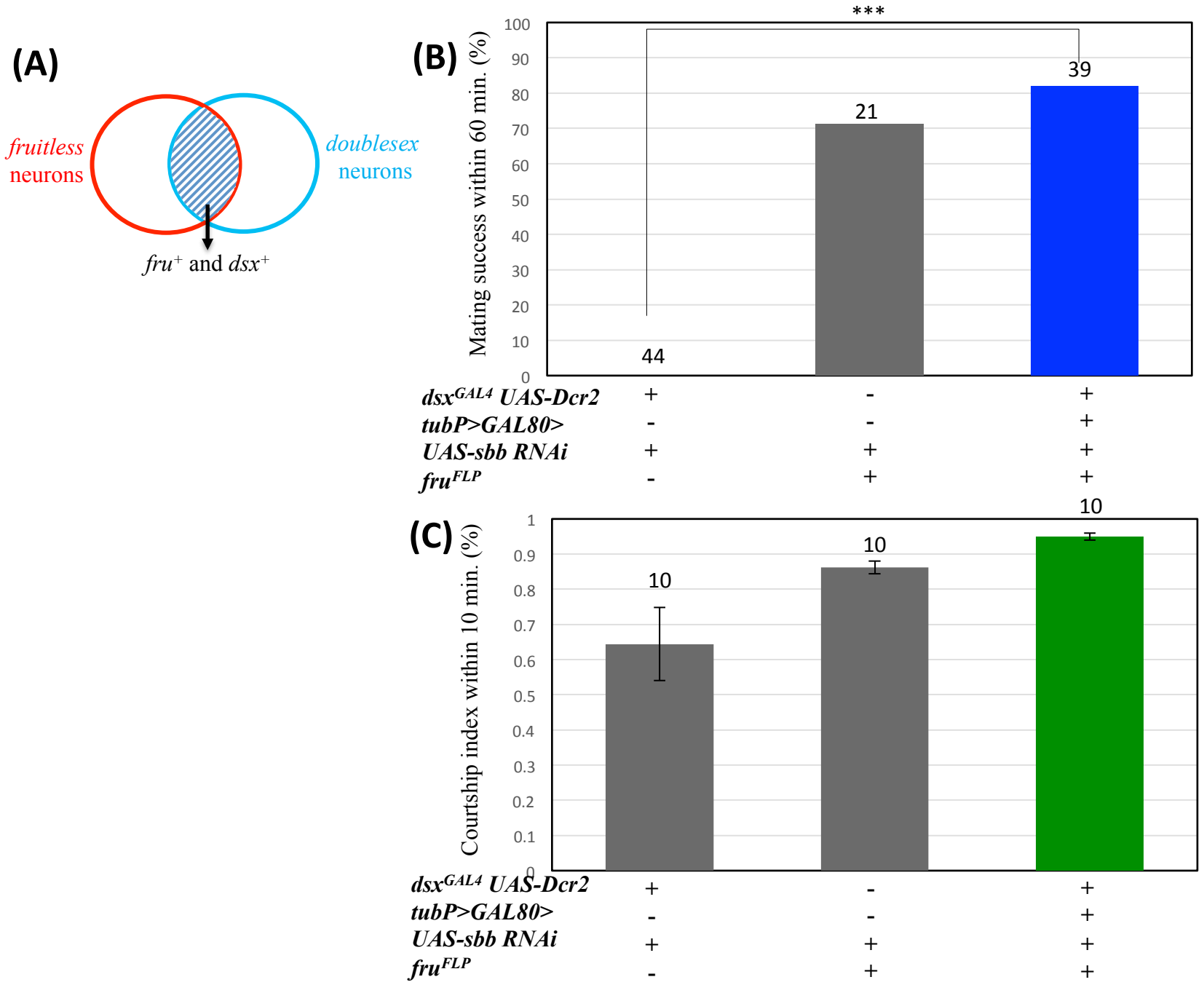
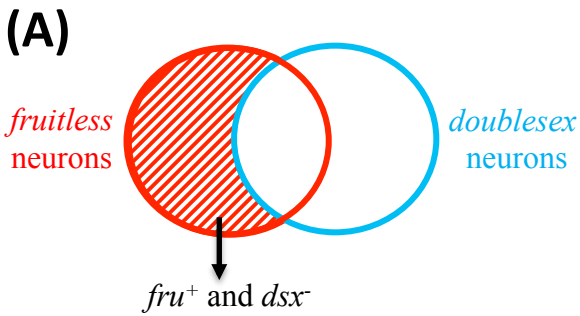

(A)
fruitless neurons
doublesex neurons
fru+ and dsx+
(B)
***
Mating success within 60 min. (%)
100
90
80
70
60
50
40
30
20
10
0
44
21
39
dsxGAL4 UAS-Dcr2 + - +
tubP>GAL80> - - +
UAS-sbb RNAi + + +
fruFLP - + +
(C)
Courtship index within 10 min. (%)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
10
10
dsxGAL4 UAS-Dcr2 + - +
tubP>GAL80> - - +
UAS-sbb RNAi + + +
fruFLP - + +

Figure 13


(A)
fruitless neurons
doublesex neurons
fru+ and dsx-

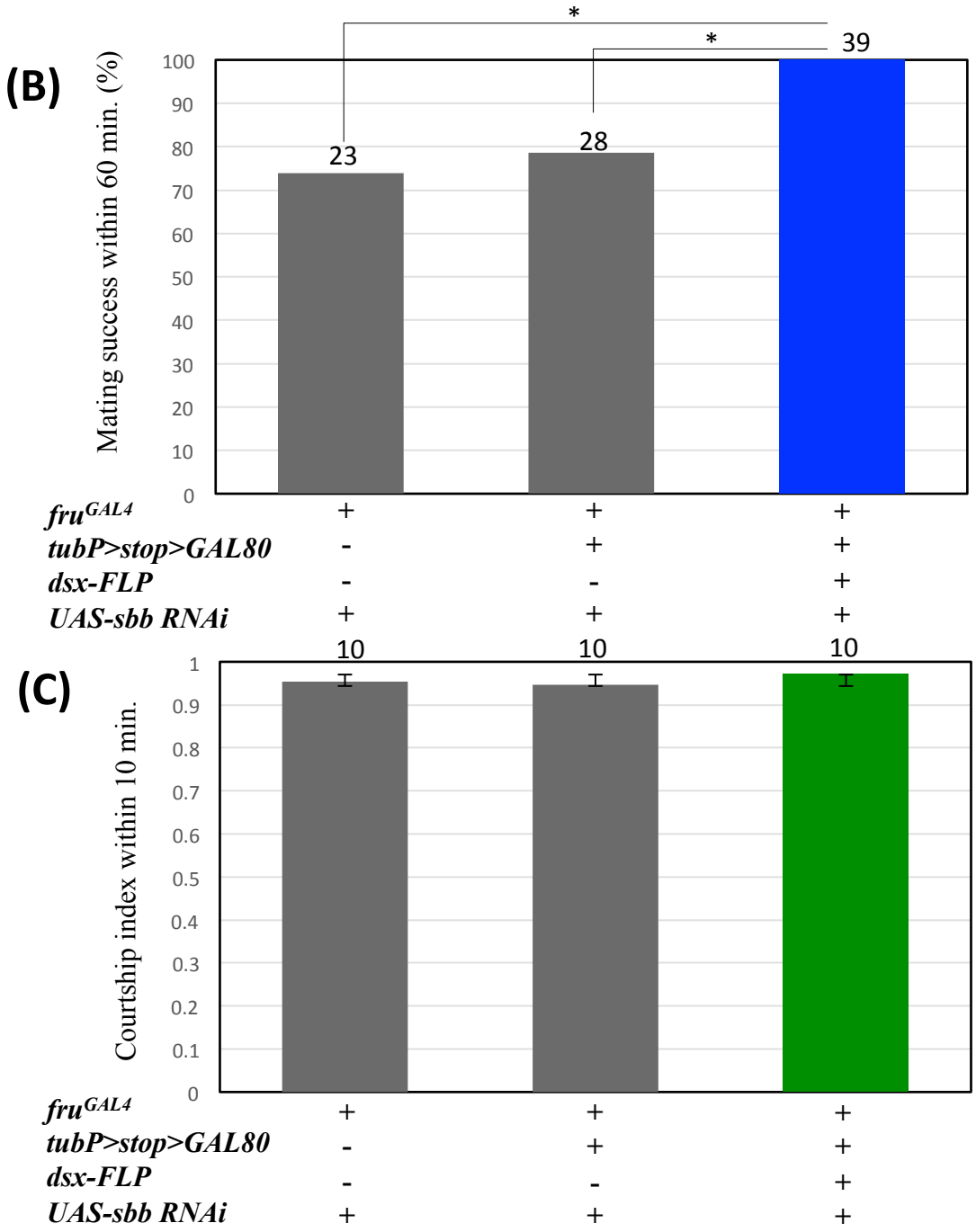
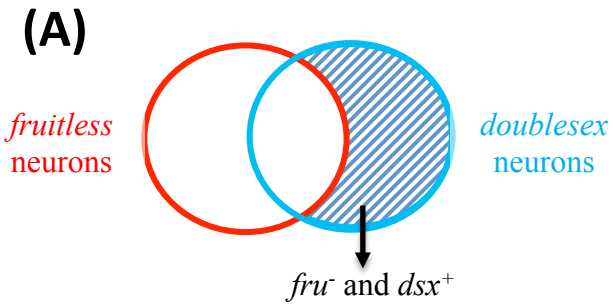
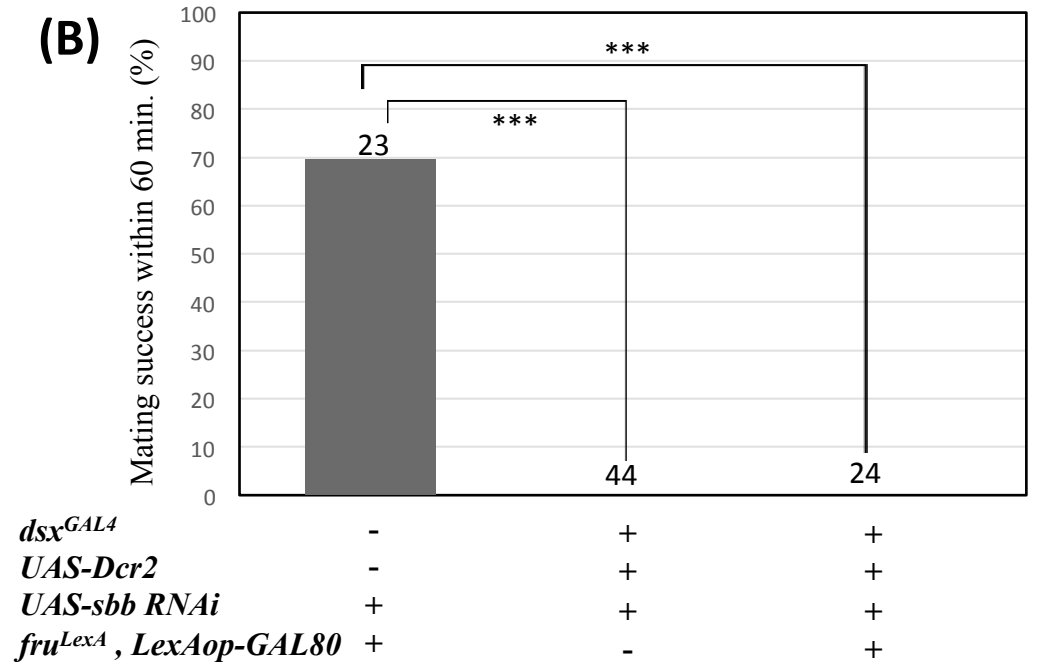

(B)
Mating success within 60 min. (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
23
28
39
fruGAL4 + + +
tubP>stop>GAL80 - + +
dsx-FLP - - +
UAS-sbb RNAi + + +
(C)
Courtship index within 10 min.
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
10
10
fruGAL4 + + +
tubP>stop>GAL80 - + +
dsx-FLP - - +
UAS-sbb RNAi + + +

Figure 14


(A)
fruitless neurons
doublesex neurons
fru- and dsx+


(B)
Mating success within 60 min. (%)
100
90
80
70
60
50
40
30
20
10
0
***
***
23
44
24
dsxGAL4 - + +
UAS-Dcr2 - + +
UAS-sbb RNAi + + +
fruLexA , LexAop-GAL80 + - +

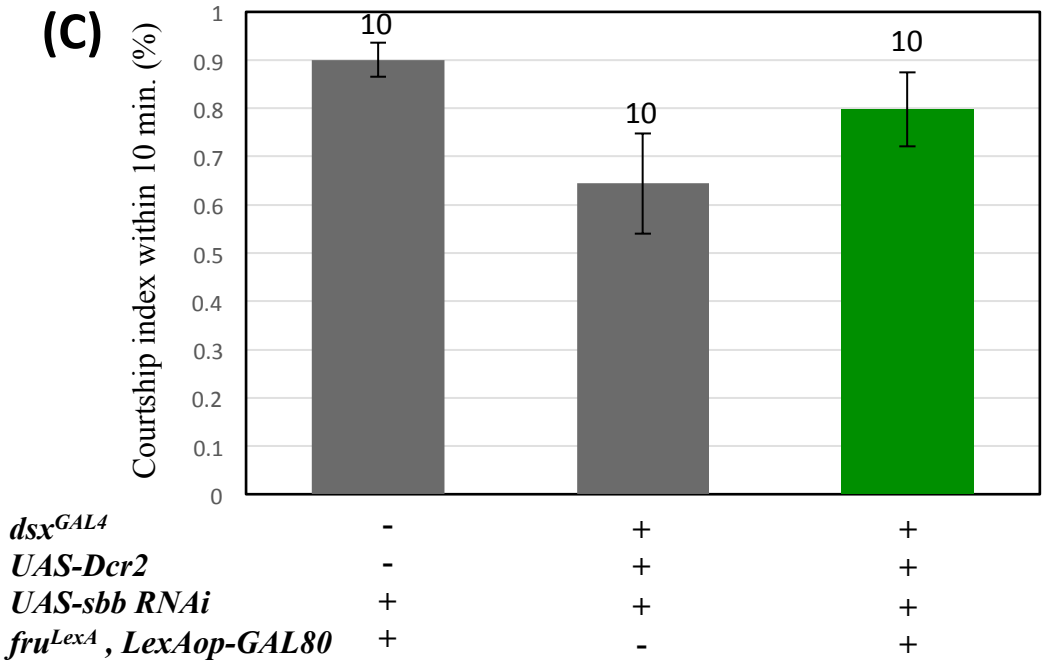
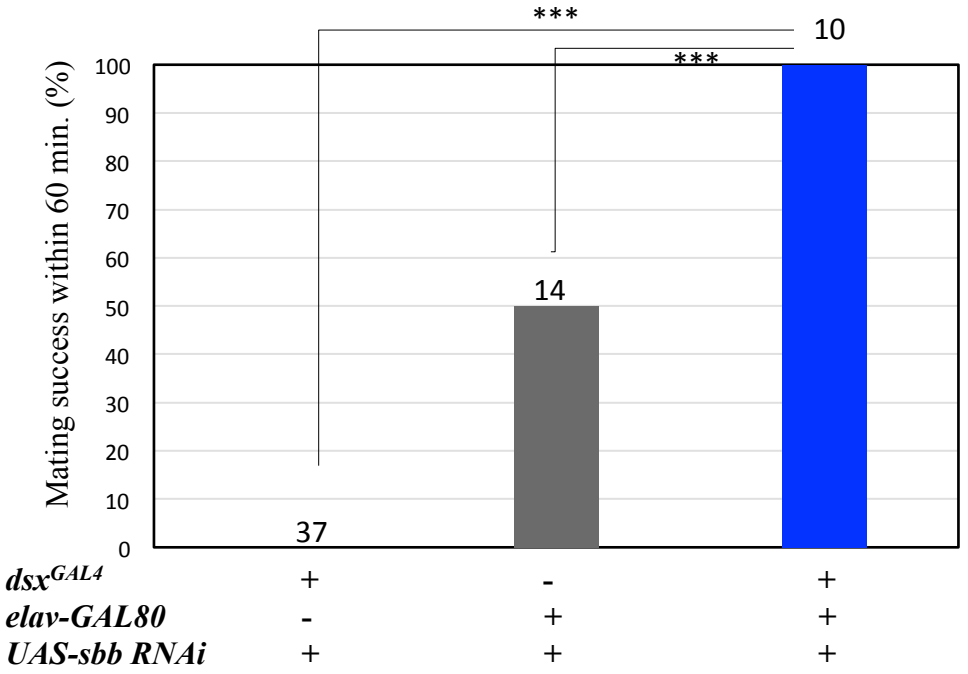

(C)
Courtship index within 10 min. (%)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
10
10
dsxGAL4 - + +
UAS-Dcr2 - + +
UAS-sbb RNAi + + +
fruLexA , LexAop-GAL80 + - +

Figure 15

(A)

(B)



(C)

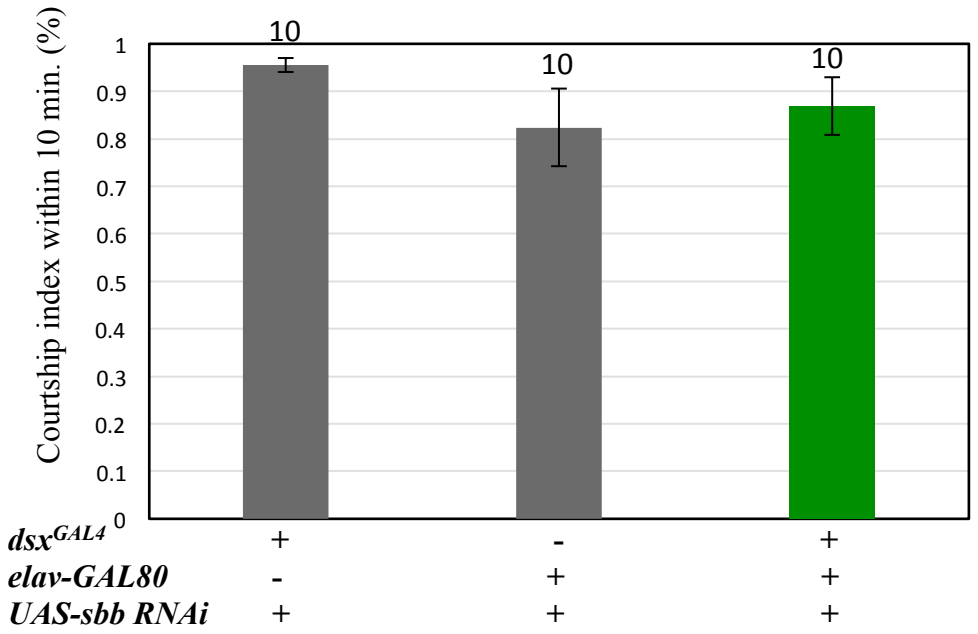


Figure 16

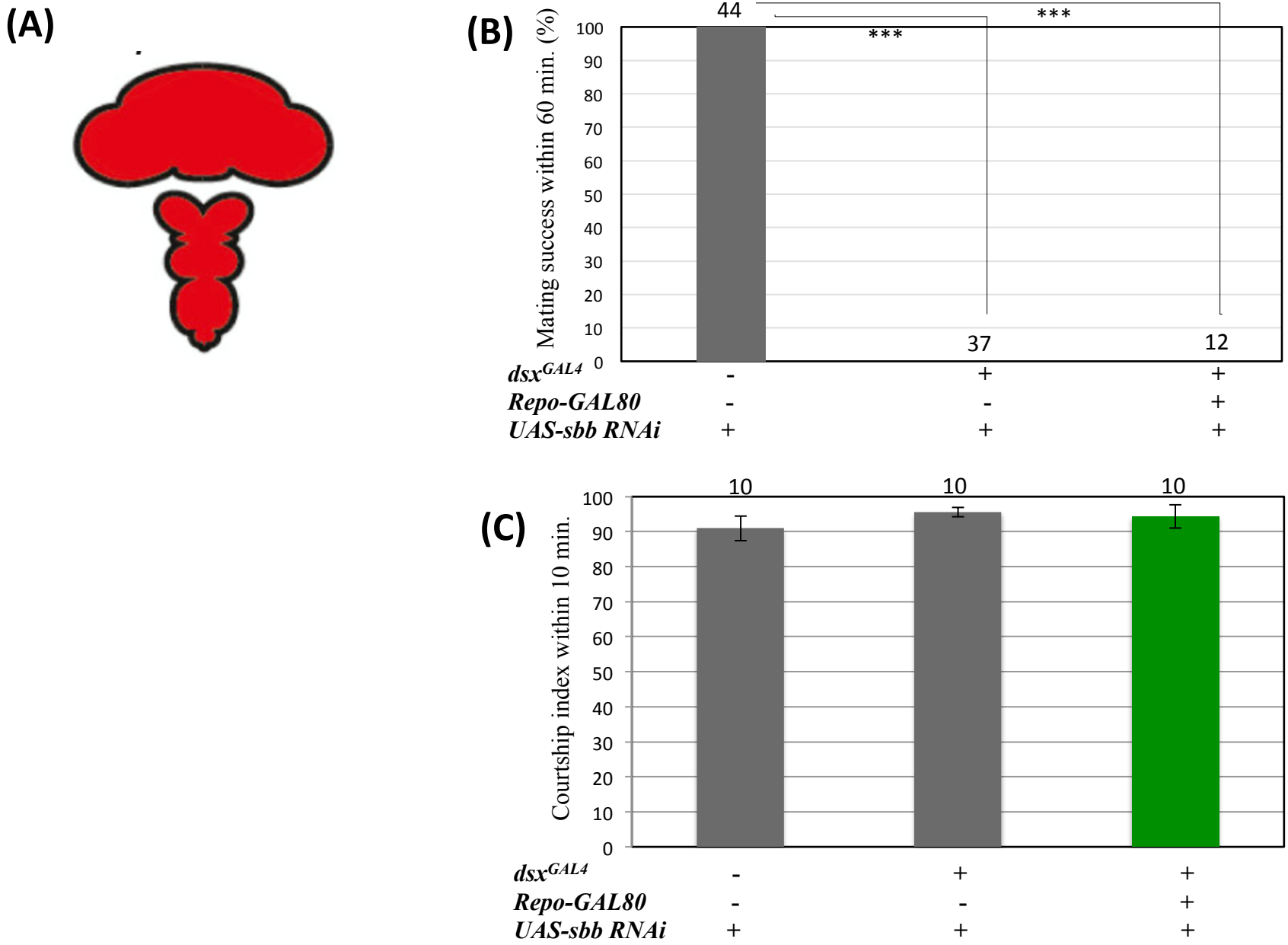
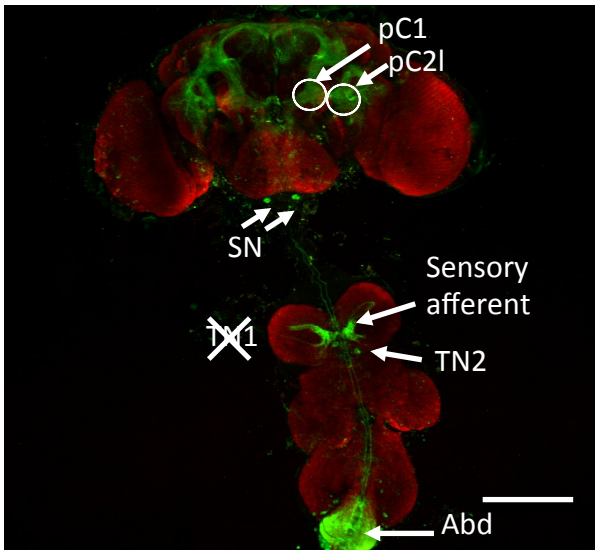

(A)
(B)
Mating success within 60 min. (%)
100
90
80
70
60
50
40
30
20
10
0
44
37
12
***
***
dsx^GAL4 - + +
Repo-GAL80 - - +
UAS-sbb RNAi + + +
(C)
Courtship index within 10 min.
100
90
80
70
60
50
40
30
20
10
0
10
10
10
dsx^GAL4 - + +
Repo-GAL80 - - +
UAS-sbb RNAi + + +

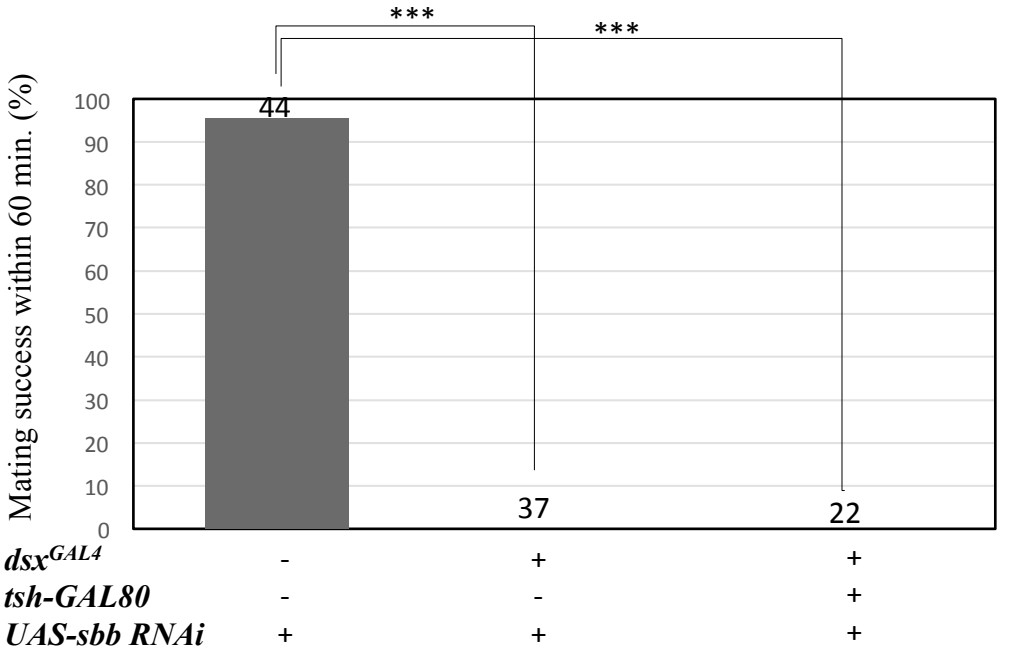
Figure 17

(A)



w; Sp/tsh-GAL80; dsx^{GAL4}/UAS-mCD8::GFP

(B)



(C)

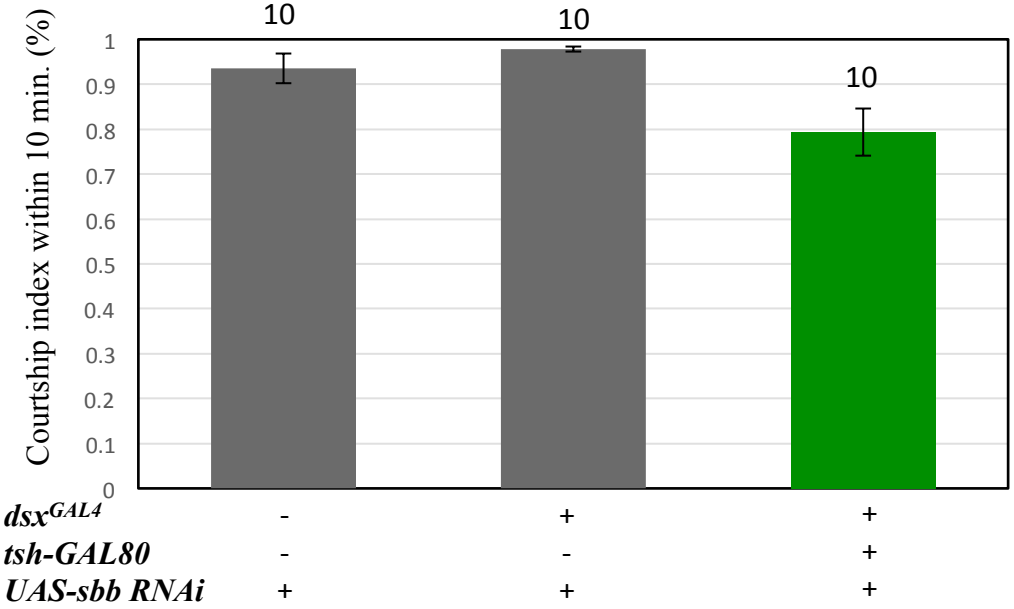
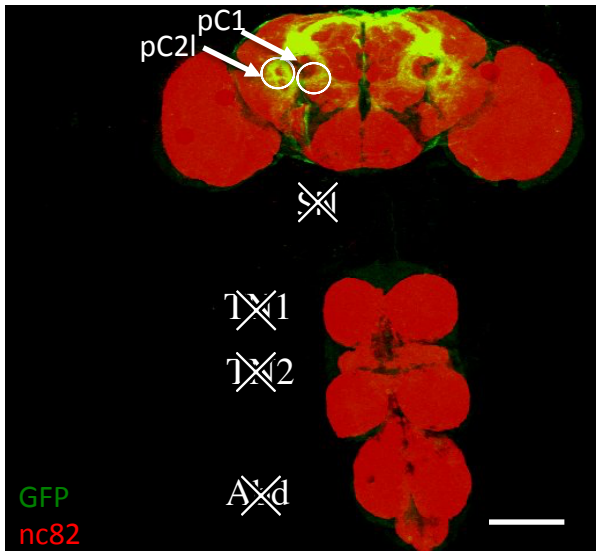


Figure 18



w; Otd-FLP/UAS-mCD8::GFP; dsx^{GAL4}/tubP>GAL80>

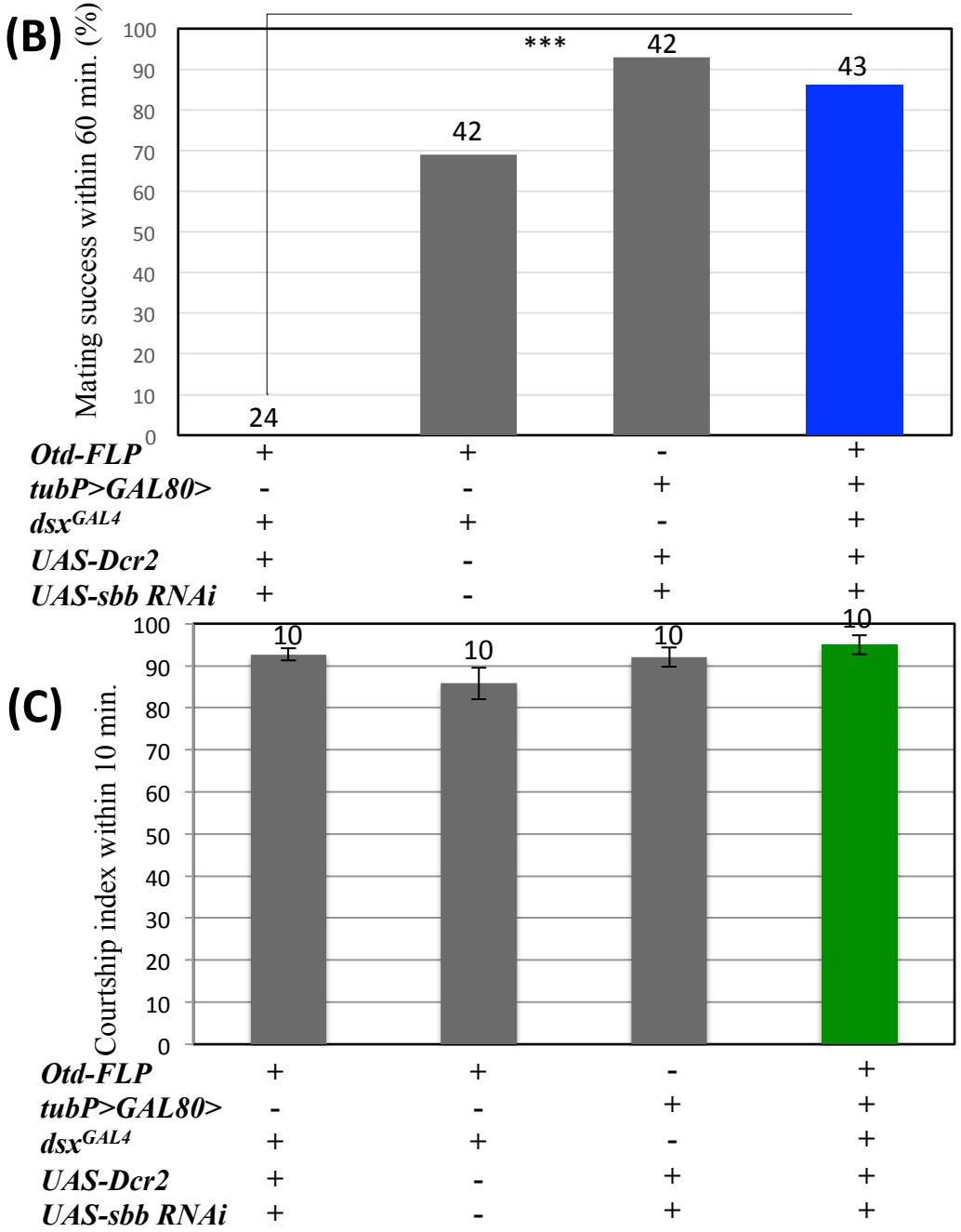
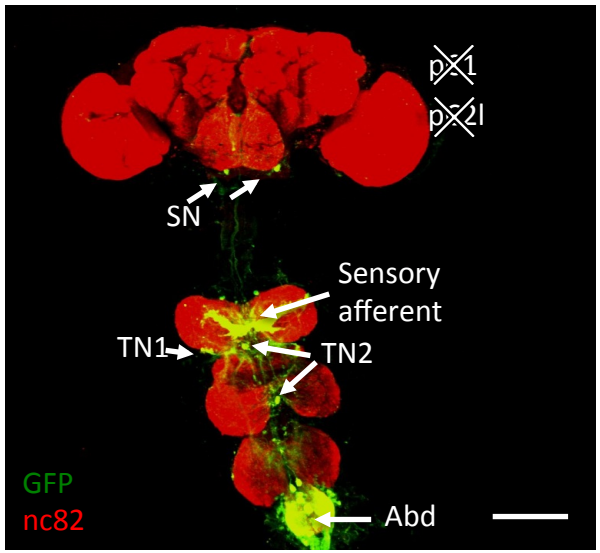


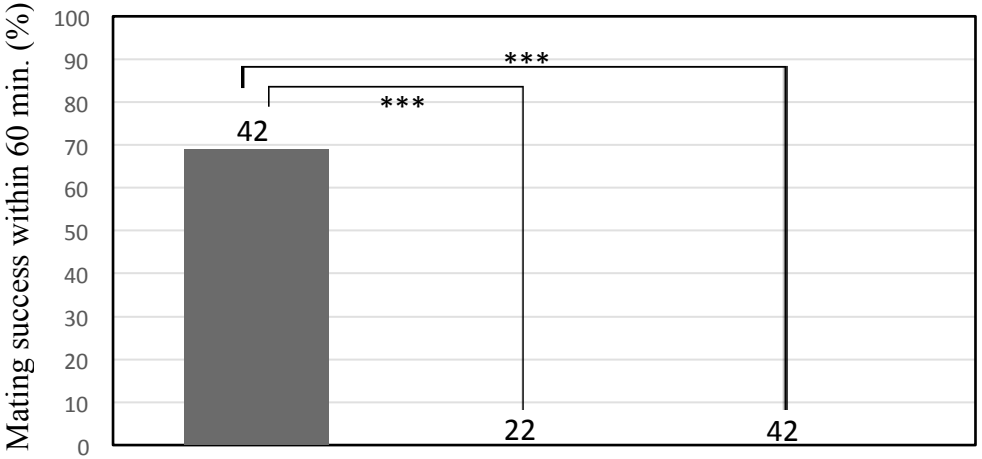
Figure 19

(A)



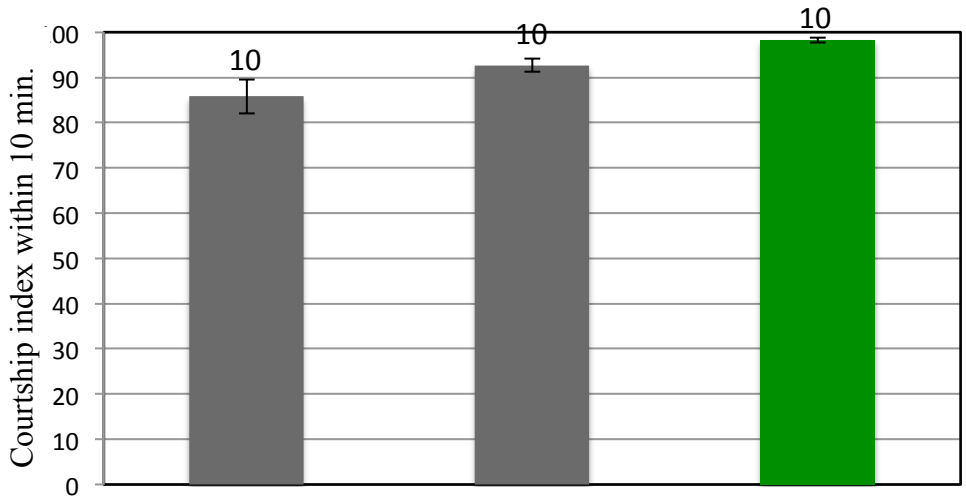
w; Otd-FLP/UAS-mCD8::GFP;
dsx^{GAL4}/tubP>stop>GAL80

(B)



Otd-FLP	+	+	+
tubP>stop>GAL80	-	-	+
dsx^{GAL4}	+	+	+
UAS-sbb RNAi	-	+	+

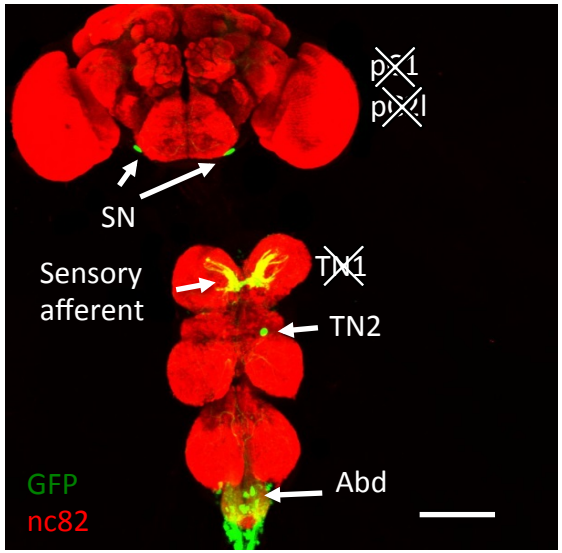
(C)



Otd-FLP	+	+	+
tubP>stop>GAL80	-	-	+
dsx^{GAL4}	+	+	+
UAS-sbb RNAi	-	+	+

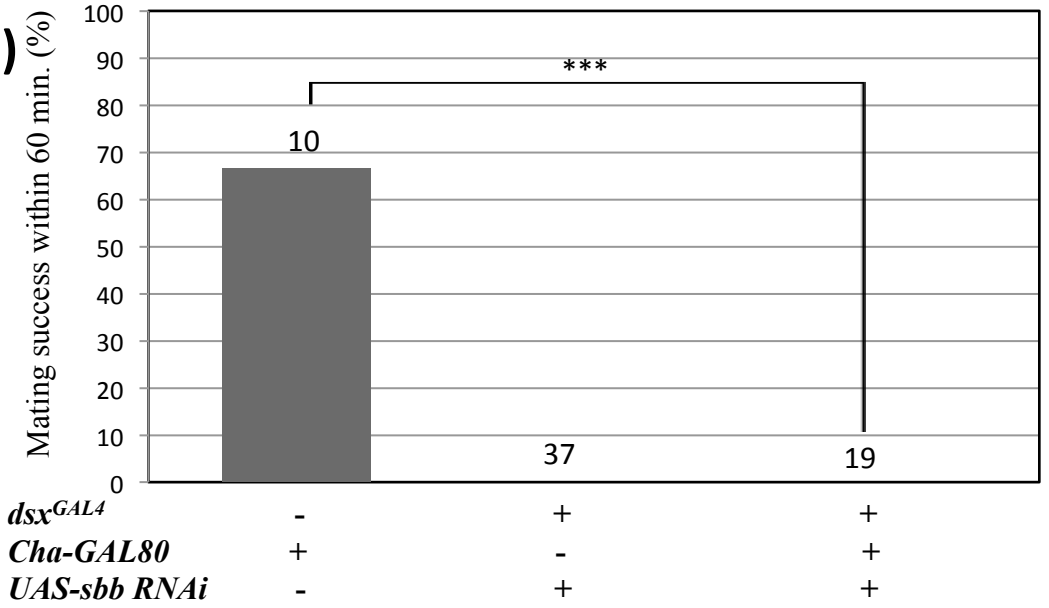
Figure 20

(A)



w;dsx^{GAL4} .UASGCamp/ Cha-Gal80

(B)



(C)

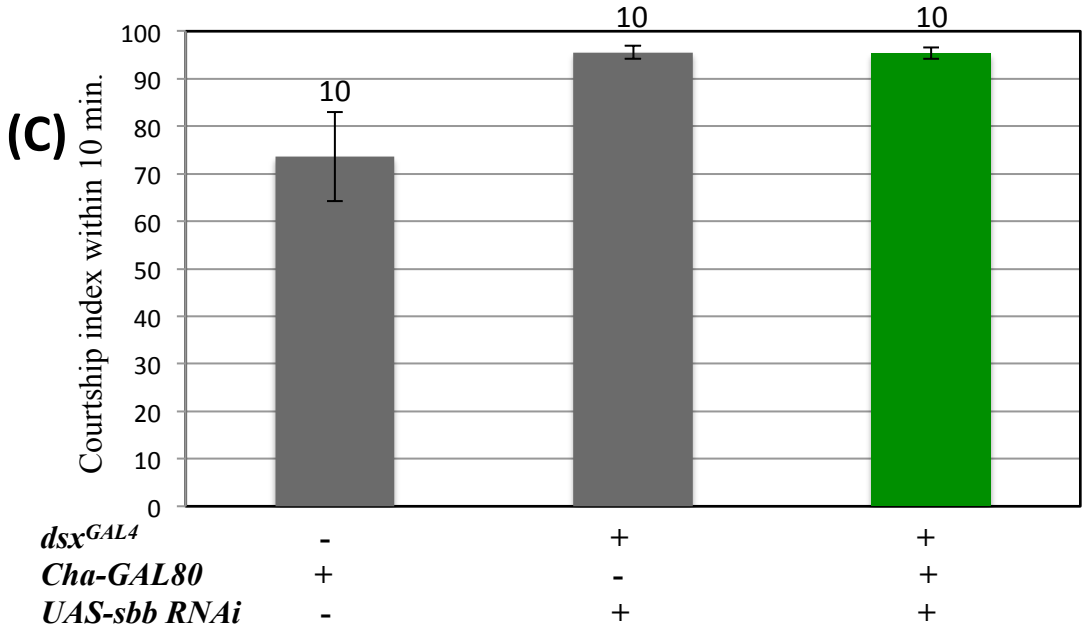
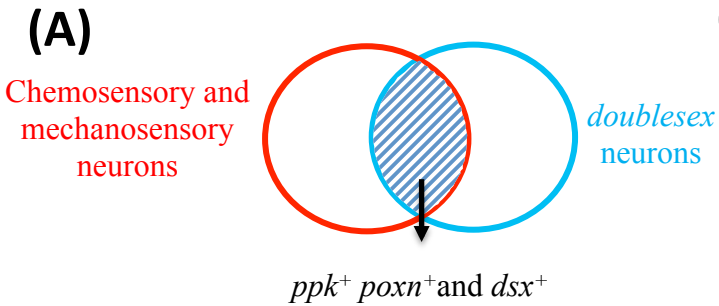
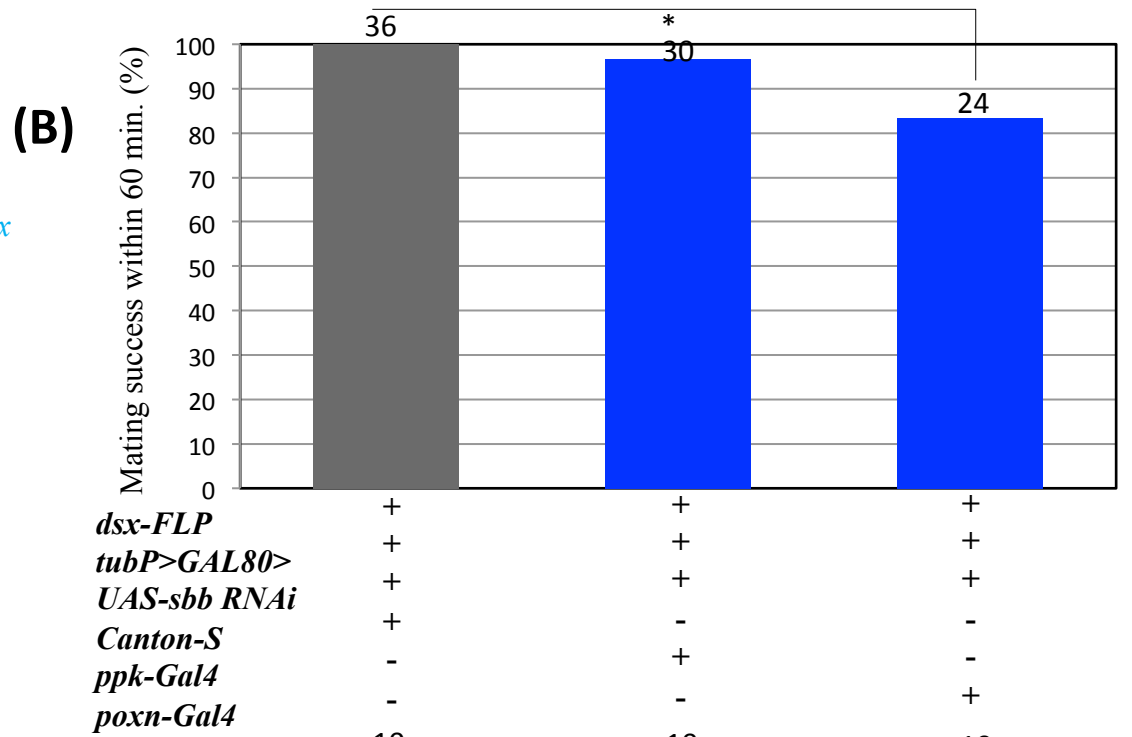


Figure 21


(A)
Chemosensory and mechanosensory neurons
doublesex neurons
ppk+ poxn+and dsx+


(B)
Mating success within 60 min. (%)
36
30
24
*
dsx-FLP
tubP>GAL80>
UAS-sbb RNAi
Canton-S
ppk-Gal4
poxn-Gal4

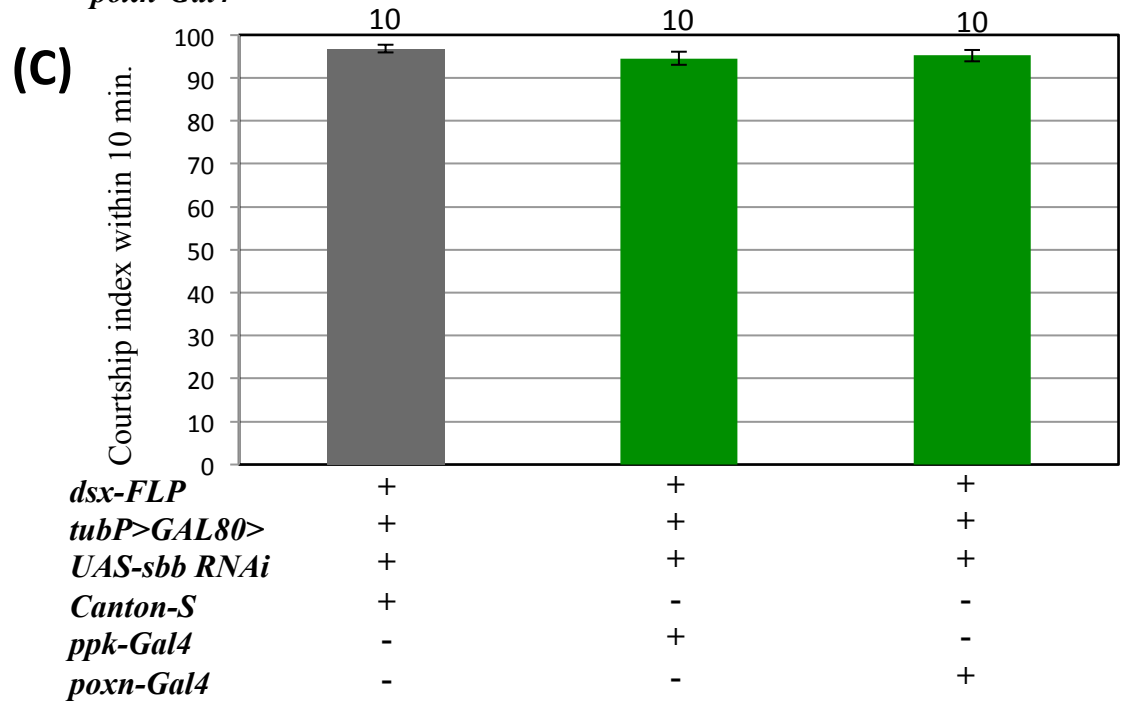
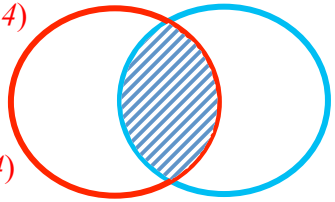

(C)
Courtship index within 10 min.
10
10
10
dsx-FLP
tubP>GAL80>
UAS-sbb RNAi
Canton-S
ppk-Gal4
poxn-Gal4

Figure 22

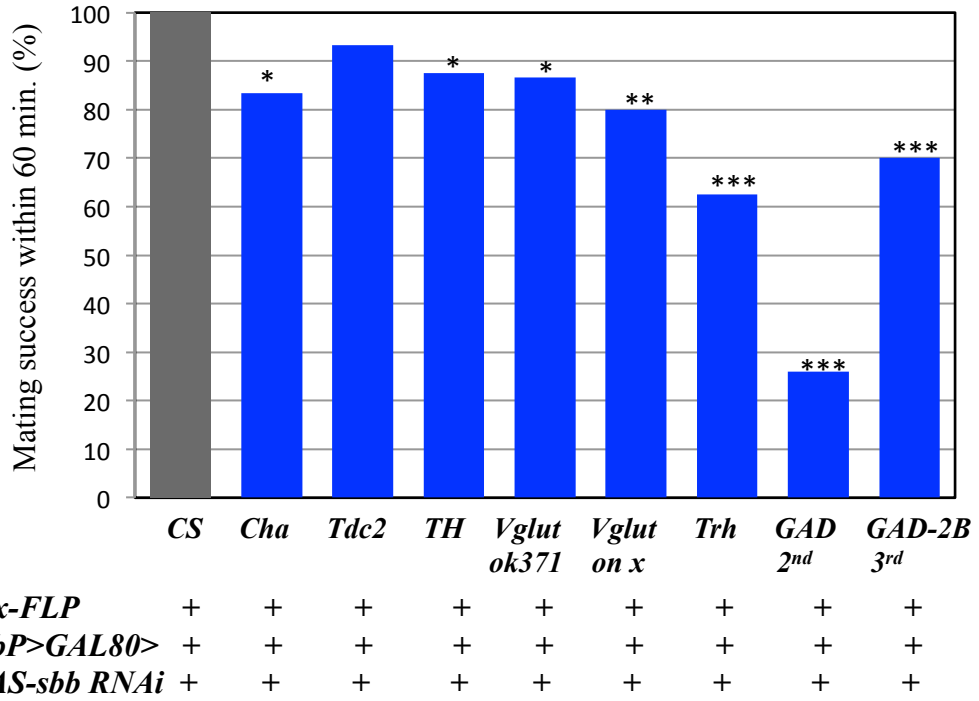
(A)

Acetylcholine (*Cha-GAL4*)
Dopamine (*TH-GAL4*)
GABA (*GAD-GAL4*)
Glutamate (*Vglut-GAL4*)
Octopamine (*Tdc2-GAL4*)
Serotonin (*Trh-GAL4*)



doublesex neurons

(B)



(C)

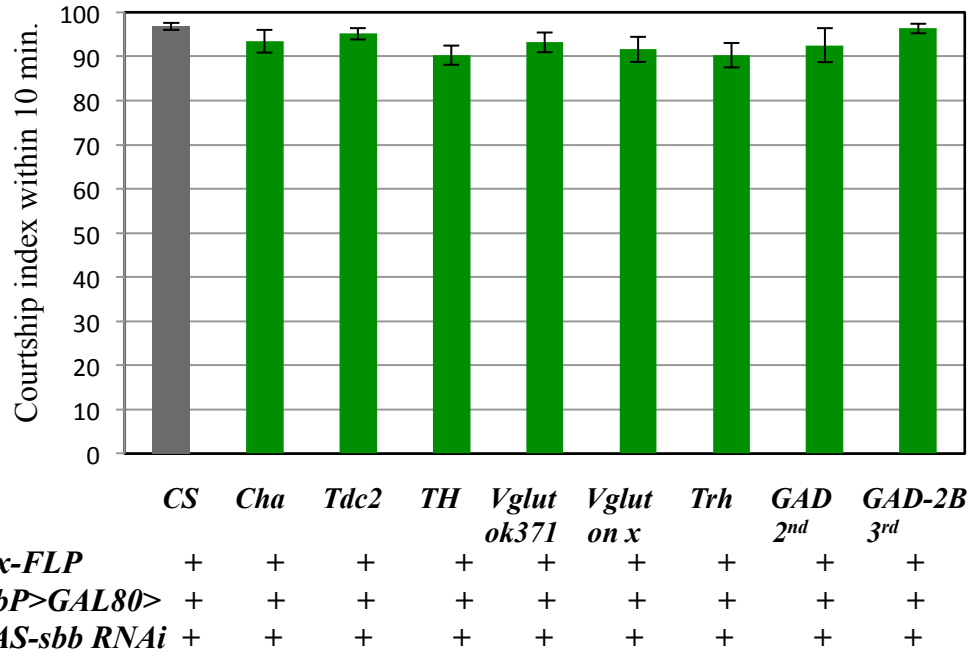
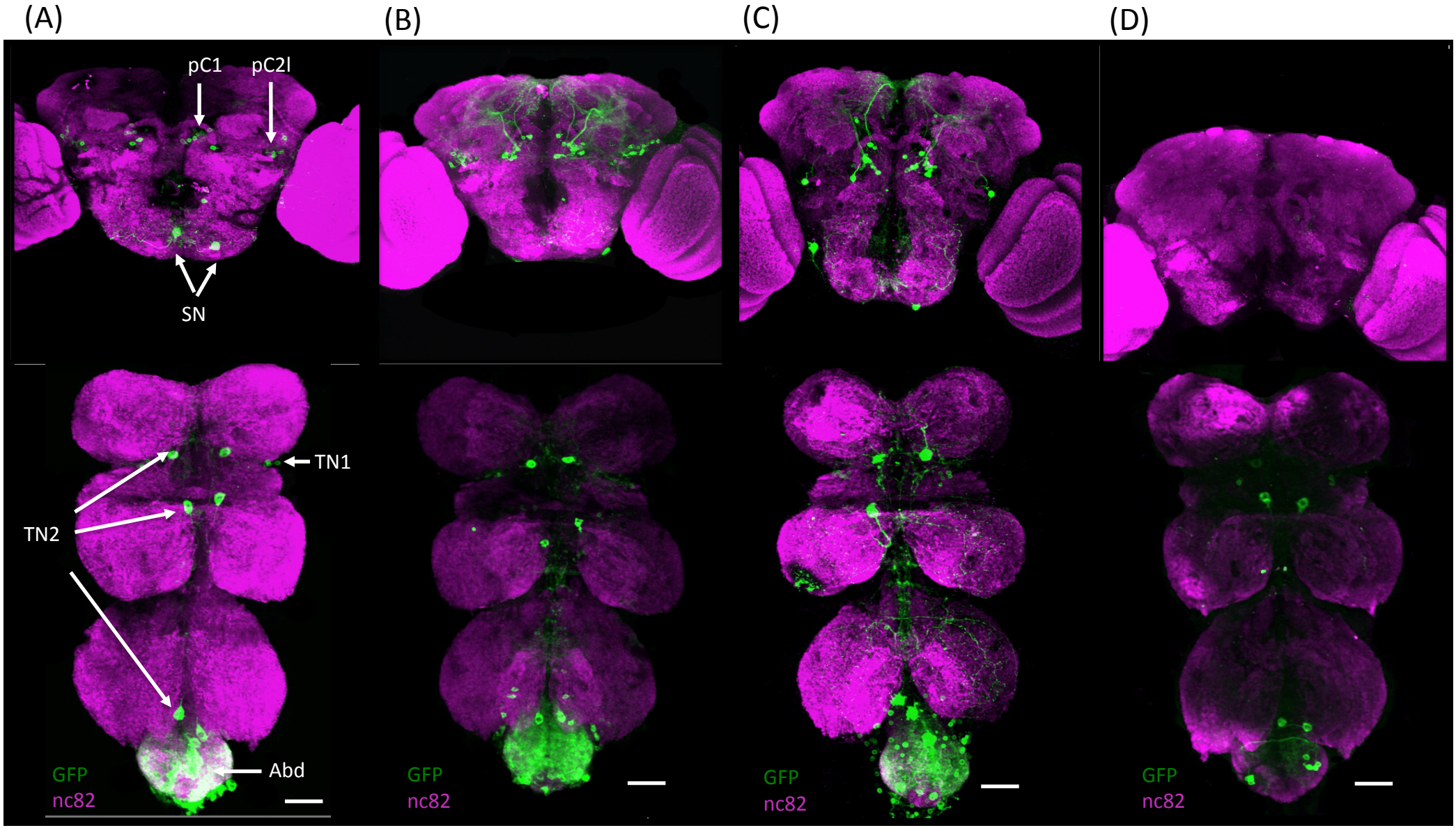


Figure 23



Vglut GAL4; UAS>stop>MCD8::GFP/dsx^{FLP}

w;UAS>stop>MCD8::GFP/ GAD-GAL4(II);dsx^{FLP}

w;UAS>stop>MCD8::GFP; GAD-2B GAL4(III)/ dsx^{FLP}

w;UAS>stop>MCD8::GFP/ Trh-GAL4;dsx^{FLP}